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RCS MEDDH - 288 (RI)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

Including

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

ANNUAL PROGRESS REPORT

1 July 1964 - 30 June 1965

VOLUME 1

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

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AD

RCS MEDDH-288 (R1)

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

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

Annual Progress Report
1 July 1964 - 30 June 1965

Volume 1

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

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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."

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PROJECT 3A013001A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6477	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / BRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 095

10b PRIOR NUMBER/CODE: None

11. TITLE: (U) Biochemical action of trace substances--effects of trace metals on hormone and enzyme activity (09)

12. SCIENTIFIC OR TECH. AREA: 002300 Biochemistry; 012900 Physiology

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
05 63	NA	OTHER | DA

16. PROCURE. METHOD: C. IN-HOUSE

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

18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
PRIOR FY		
CURRENT FY		

19. GOV'T LAB/INSTALLATION/ACTIVITY
NAME: Headquarters
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RESP. INDIV.: Tigertt, Col. W.D.
TEL.: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Mertz, Dr. W.; Roginski, E.R.; Glinsmann, Dr. W.
TEL: 202-576-3527 TYPE: DA

21. TECHNOLOGY UTILIZATION: Food technology

22. COORDINATION: NA

23. KEYWORDS: Chromium; metals; diabetes; glucose; arteriosclerosis; insulin; fatty acids; metabolism; nutrition

24. (U) Tech Objective - To define the mode of action of chromium (III) in metabolism; to establish the nature of its interaction with insulin and related substances; to determine the metabolic defects in chromium deficiency in animals and man and to correct such defects by adequate supplementation.

25. (U) Approach - Aspects of glucose, protein and fat metabolism are investigated in chromium-deficient rats and in diabetic patients, in collaboration with Schroeder, (Brattleboro) and with 3 medical schools. In vitro tests are performed for biological activity of specially prepared complexes of chromium with biological materials to determine the influence of chemical structure on biological activity.

26. (U) Progress (Jul 64-Jun 65) - Studies on total-body chromium metabolism have been completed. A new, small chromium compartment has been found with a fast turnover which is strongly influenced by glucose and insulin. Rats raised under strictly controlled low chromium conditions exhibited a nearly diabetic glucose tolerance; this defect was repaired by Cr supplementation. Some human diabetics were significantly improved by Cr treatment, others were not able to absorb the element. A clinical test on a large scale has begun. Chromium appears to be required for a stimulation of fatty acid oxidation by insulin in heart and aorta. These experiments are still in progress.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 095, Biochemical action of trace substances -- effects of trace metals on hormone and enzyme activity

Investigators.
Principal: Walter Mertz, MD
Associate: E. E. Roginski; Capt Walter H. Glinsmann, MC; Major Richard C. Reba, MC; Capt John Freeman, MC; Angel J. Pentschew, MD

Description. A potentiating effect of trace amounts of trivalent chromium on the action of insulin has been established in many systems. This effect has been previously studied in detail with regard to carbohydrate metabolism. At the present time, other nutrients are under investigation as substrates in vitro.

These studies are being correlated with in vivo experiments in which the effect of trace amounts of chromium on aging processes and on response to various forms of stress is determined. Aspects of chromium metabolism as well as the effect of chromium supplementation are being studied in human diabetics.

Manganese toxicity in man is known to produce liver damage as well as a syndrome of basal ganglia disease with specific topistic involvement of the central nervous system. The intent of this investigation is to produce this syndrome in small laboratory animals and to utilize these animals to study the mechanism of action of manganese.

Progress. Chromium Studies - It is difficult to induce and maintain even a marginal chromium deficiency under ordinary conditions. This is apparently due to difficulty in control of external contamination of diet, water, and air. The hypothesis that subjecting animals to various forms of stress may lead to an increased chromium requirement -- and thus to a more pronounced deficiency was tested, using the nutritional stress of low-protein diets. Rats raised on these suboptimal rations and kept in plastic cages improved their growth rates significantly when supplemented with chromium in their drinking water. This effect became more pronounced when the animals were subjected to a measured amount of exercise. When blood was acutely withdrawn (3% of body weight), only 8% of chromium-supplemented animals died, as compared with 33% of controls. Similarly, recovery of the weight loss after hemorrhage was approximately four times that of chromium-

deficient controls. These experiments are now being repeated; other long-term studies are still in progress. In the latter, the appearance of eye lesions, probably corneal defects, in the chromium-deficient, but not in the supplemented groups, was observed.

In another group, kept for longevity studies, 3 out of 10 chromium-deficient rats are surviving after 20 months, as compared with 8 of 10 in the chromium-supplemented rats.

The mechanism leading to increased growth rates in the chromium animals is being studied in vitro. The weight gain is not due to increased protein. Preliminary results suggest that chromium promotes the active transport of amino acids into the cell. The incorporation of intracellular amino acids into cell protein appears not to be significantly changed by chromium. These observations again point to a membrane mechanism, which is also insulin-sensitive, as a site of action of chromium.

Studies dealing with the metabolism of intravenously injected chromium have been finished. Doses of 0.05-0.1 micrograms of chromium /100g are sufficient to improve impaired intravenous glucose tolerance of chromium-deficient rats and increase the response of their epididymal fat tissue to insulin. Acute toxicity is observed only with 10,000 times these doses. The metabolism of physiological amounts of the element was investigated with the use of chromium-51. The handling of chromium by the organism is unaffected by previous dietary history and amounts given. Absorption from the gastro-intestinal tract is in the range of a few per cent of an oral dose. Elimination of whole-body activity can be described by a multiexponential expression. Three distinct components can be calculated, with half lives of 0.5, 5.9, and 83.4 days, respectively. The efficiency of this excretion system may account for the observation that chromium does not accumulate in the body to any significant degree, even when fed during the whole life span of animals.

During these studies, a new aspect of chromium metabolism was discovered which is being investigated in experimental animals and in man. Plasma chromium rises acutely to up to five times its original concentration following glucose administration to diabetics treated with chromium. This rise is also seen in chromium-sufficient rats, where it can be elicited not only by glucose but also by insulin injections. The significance of these findings is presently unknown. Further experiments are in progress.

In collaboration with Dr. Henry Schroeder, Brattleboro, Vermont, a study on the effect of chromium-deficiency on glucose metabolism was concluded and published. Rats rasised under conditions allowing strict control of external trace element contamination, but supplemented

with all trace elements recognized as essential, exhibit a diabetic type intravenous glucose tolerance. In some animals, no glucose removal from the blood was observed at all, during one hour. Chromium supplementation improved this condition toward normal, and it resulted in a significantly greater response of epididymal fat tissue to graded levels of insulin.

Studies on the role of chromium in fat metabolism were initiated and are still in progress. Preliminary results indicate that in heart slices as well as in aorta strips, but not in brain, liver, diaphragm, spleen and fat tissue, the oxidation of palmitate is stimulated to a small degree by insulin in vitro. Addition of chromium results in a significant 30% increase over the control. The effect is dependent on the presence of glucose in the medium. Both of these actions were observed during fall and early winter only. The system is being followed weekly, to establish whether seasonal variations are involved, as has been reported for other aspects of lipid metabolism. A potential significance of these studies may arise, in the light of the established decline of chromium concentrations with age, in heart and aorta of the U. S. population.

Clinical studies have been finished. Two more patients with mild diabetes were treated with chromium, while being kept on a metabolic ward under strictly controlled conditions. Both improved significantly with the chromium supplement. One patient who could be observed intermittently through one year, went through 3 subsequent cycles of treatment, with control periods between. In each treatment period, a significant improvement was seen. These studies have now been transferred to a larger scale. Clinical trials are under way in five different departments, where the potential value of chromium in the treatment of diabetics is assessed independently.

Manganese studies - Newborn rats have been injected daily with varying dosages of manganese. A critical dosage has been found which profoundly affects the growth and weight gain of these rats and also produces profound liver changes in addition to changes within the central nervous system. An investigation of the pathologic nature of these changes in the liver and brain has been undertaken in conjunction with Dr. Pentschew of AFIP. Manganese-54 is being utilized to investigate the reason for the narrow critical range of toxicity as well as to study localization of the chronically injected manganese to organs of the body and the specific intraorgan localization. This latter study will utilize radioautography.

It is planned to study the effect of manganese on the oxygen consumption and glucose utilization of the liver and the brain and the liver-brain interrelationships.

Summary and Conclusions. In chromium-deficient rats, the effect of insulin is impaired in many systems. Chromium-deficient rats, raised in a strictly controlled environment exhibit severely impaired glucose tolerance, which is improved by trace amounts of the element. Chromium supplementation of low-protein rations enhanced growth, prolonged survival and increased resistance against death from hemorrhage. Preliminary data suggest these effects may be mediated through an interaction of chromium with insulin at the membrane. Chromium significantly enhanced the stimulation of palmitate oxidation by insulin in heart and aorta slices. This effect was observed in fall and winter only. It is being followed. The acute toxic level of chromium(III) is 10,000 times the physiologically active dose. Physiological amounts are excreted according to a set of first-order reactions. In carefully controlled long-term experiments, three mild diabetics have responded to chromium supplementation with a significant improvement of glucose tolerance. Lesions have been produced by manganese injections in liver and brain of newborn rats.

Publications.

1. Mertz, W., E. E. Roginski and H. A. Schroeder: Some Aspects of Glucose Metabolism of Chromium-Deficient Rats Raised in a Strictly Controlled Environment. J. of Nutrition 86, 107-112, 1965.

2. Mertz, W. and W. H. Glinsmann: The Effect of Trivalent Chromium on Glucose Utilization in Diabetes Mellitus. Excerpta Med. 74, 137, 1964.

3. Roginski, E. E. and W. Mertz: Effect of Insulin and Chromium(III) on Palmitate Oxidation. Fed. Proc. 24, 510, 1965.

4. Harlow, D. R., W. Mertz and J. F. Mueller: Effects of Spirometra Mansonoides Infection on Carbohydrate Metabolism. An Insulin-Like Effect from the Sparganum. J. of Parasit. 50, 53, 1964.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6478	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A01300191C 01 096

10b. PRIOR NUMBER CODE: None

11. TITLE: (U) Neutron-induced radioactivity in subsistence (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
013900 Radioactivity: 011500 NUCWPN eff; 014000 rdo & radn chem.	06 63	NA	OTHER / DA

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

19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL.: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012
INVESTIGATORS: Wampler, Capt S.N.
PRINCIPAL ASSOCIATE: Ellis, M.K.
TEL: 202-576-3646 TYPE: DA

21. TECHNOLOGY UTILIZATION: Irradiation sterilization of food neutron

22. COORDINATION: NA

23. KEYWORDS: Food contamination; food irradiation; radiation; radioactivity, neutron induced phosphorus 32; sulfur 35

24. (U) Tech Objective - The principal objective of this project is to define, quantitate and seek empirical equations to demonstrate the hazard of neutron-induced radioactivity in food subsistence that might be expected from weapon explosions on the battlefield. The investigations encompass the correlation of experimental results with calculated values and development of analytical methodology for the investigation.

25. (U) Approach - The plan is to expose foods in the nuclear reactor to neutron fluxes similar to those exposures expected under battlefield conditions. Following exposure samples are chemically analyzed and separated for radiation counting and the results compared with calculated values for that exposure.

26. (U) Progress (Jul 64 - June 65) - A total of 236 samples of subsistence and control standards were exposed in the WRAIR reactor at an approximate thermal neutron flux of 10^{13} nvt. The samples allowed the problem of S^{35} and P^{32} to be defined and quantitated. Results indicate that standard activation equations are satisfactory to be utilized as guides for the definition of the hazard of neutron induced P^{32}, S^{35} and Ca^{45} in subsistence.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

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ANNUAL PROGRESS REPORT

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 096, Neutron-induced radioactivity in subsistence

Investigators.
Principal: Capt Stanley N. Wampler, VC
Associate: Mary K. Ellis, B.S.; Capt Charles G. Liddle, VC

Description.

The problem of neutron induced activity by high thermal neutron fluxes is being studied to provide information for establishing guidelines for the handling and distribution of military subsistence items exposed to low yield nuclear weapons. Studies are being made to establish the levels of induced radioactivity to be expected and to devise and standardize methods for detecting and quantitating levels of the more important radionuclides.

Progress.

For the determination of sulfur-35, a total of 278 samples of subsistence and control standards were exposed in the Walter Reed Research Reactor at thermal neutron fluences ranging from 10^9 to 10^{13} nvt. The amount of stable sulfur was determined using classic analytical methods by the precipitation of sulfur as barium sulfate. The first control standards used were ammonium sulfate. From preliminary studies, the resultant activity of the sulfur standards versus the activity of the subsistence samples containing the same amount of sulfur led to other parameters of consideration and, as a result, stable chlorine determinations were made for each subsistence item using ammonium chloride standards as controls.

Ammonium chloride standards containing the same amount of stable chlorine as the subsistence samples were activated with the samples and used to calculate the flux of the reactor for each irradiation as an instantaneous dose. The activity of the ammonium chloride standards closely correlated with the activity of the irradiated samples.

Subsistence items used for the sulfur-35 determinations were peanut butter, dried eggs, and canned ham. The canned ham contained the highest per cent of chlorine, while both canned ham and dried eggs contained about the same amount of sulfur. The results of one series of activated samples are seen in the table below:

Subsistence	% S ***	% Cl ***	Fluence (10^{13})*	NH_4Cl (g)**	S^{35} Calculated (μc)	S^{35} Experimental (μc)
Canned Ham	0.146	1.52	1.20	0.1099	1.19×10^{-2}/4g	1.30×10^{-2}/4g
Peanut Butter	0.036	0.89	1.61	0.0680	7.03×10^{-3}/4g	5.20×10^{-3}/4g
Dried Egg	0.148	0.65	1.78	0.0204	2.50×10^{-3}/2g	2.29×10^{-3}/2g

* calculated using the NH_4Cl** as the standard
** irradiated
*** stable isotope

Because of the abundance (4%) and the small cross section of sulfur-34 as opposed to the greater abundance (75%) and larger cross section of chlorine-35 for production of sulfur-35, it can be concluded that the amount of stable sulfur can be neglected at integrated flux ranges of 10^9 to 10^{13} nvt. The activity of sulfur-35 from stable sulfur is one-hundredth (10^{-2}) that of the activity from stable chlorine and, therefore, would be within the 20% error of flux measurement. This is of particular interest since most foods contain chlorine--an element of salt.

Some initial counting was performed using a GM system, but was abandoned because of the poor counting efficiency (5%) and low levels of activity. The final method of counting was with a liquid scintillation counting system with an efficiency of 46% ± 2%.

The barium sulfate precipitate was collected on a millipore filter. The filter was dried and placed into a counting vial. Barium sulfate is almost completely insoluble; however, the millipore filter was dissolved using dimethyl formamide. A scintillation mixture of toluene, PPO, and POPOP was added. This was followed by the addition of a thixotropic agent, Cab-o-sil, which held the barium sulfate dispersed throughout the medium by the formation of a gel. The addition of Cab-o-sil did not lower the efficiency. This was shown by counting a soluble sulfur-35 of known specific activity without Cab-o-sil and recounting after the addition of Cab-o-sil. There was no change in the count rate. The half-life and beta energy curves for the ammonium chloride standard after irradiation and the barium sulfate-35 precipitates from the irradiated subsistence samples were studied and were in agreement with the accepted values of 87 days and 0.167 MeV, respectively.

Irradiated ham samples were sent to the Army Area Laboratories to be analyzed for sulfur-35. The samples were accompanied by a sulfur-35 standard of known specific activity, a non-irradiated ham sample, and a procedure for the determination of sulfur-35, stable sulfur, chlorine, and per cent recovery. The report of analysis is seen below.

SUBSISTENCE SAMPLES #26A and 26B

Laboratory	Cl %	S %	$T_{1/2}$ (Days)	14 Jan 65 Act µc/4g (26A)	14 Jan 65 Act µc/4g (26B)
A	1.35	.066	86	1.26×10^{-2}	1.26×10^{-2}
B	1.46	.053	87.5	1.21×10^{-2}	1.01×10^{-2}
C	2.53	.115	NR	1.65×10^{-2}	2.09×10^{-2}
D	2.03	.063	86	1.8×10^{-2}	1.6×10^{-2}
E	1.47	.178	NR	4.0×10^{-3}	1.9×10^{-3}
F	1.43	.597	81	7.79×10^{-2}	2.65×10^{-2}
G	2.3	.101	79.7	1.48×10^{-2}	1.32×10^{-2}
W	2.6	.158	81	1.27×10^{-2}	1.00×10^{-2}

The calculated activity was 1.13×10^{-2} µc/4g of ham on 14 January 1965. Since the error in some reporting laboratories appeared mathematical, a summary review for the calculation of efficiency, methods for dilution of isotopes with high specific activity, and procedures for dilution for a desired count rate, along with some mathematical calculations worked in detail were sent to the Army Area Laboratories in the final report. Another study for sulfur-35 in irradiated subsistence items will be made within the Army Area Laboratories at a later date.

Other isotopes submitted to the Army Area Laboratories were yttrium-90, iron-59, and calcium-45. The laboratories showed exemplary capabilities for the identification of the isotopes as unknowns and for the determination of specific activities of the isotopes. Further collaboration with the Army Area Laboratories will be made to continue their development and maintain their proficiency. A statistical report of analyses will be attempted.

Application of the procedure for the determination of radiophosphorus-32 as reported in the Annual Progress Report, Walter Reed Army Institute of Research, 1 July 1963 - 30 June 1964, page 652, was used successfully for the determination of induced phosphorus-32 in bone to determine flux levels in a nuclear incident during the past year.

Summary and Conclusions.

Studies on induced activity of foods employing the Walter Reed Research Reactor were continued. Laboratory analyses for radioactive sulfur-35 were reproducible within the errors of measurement. The liquid scintillation method of counting was used for greater efficiency, thus higher count rate, using a thixotropic agent to suspend the precipitate of barium sulfate.

Evaluation studies were continued with the Army Area Laboratories. Several isotopes were submitted for identification. A first attempt for the sulfur-35 activity in irradiated ham samples was commendable. These evaluation studies are being continued with new or revised procedures being sent to the Army Area Laboratories.

Neutron induced activity studies are being continued on subsistence and analysis for gamma emitting isotopes will be initiated using gamma spectrum analysis.

Publications.

Ellis, M. K., Wampler, S. N., and Yager, R. H. Determination of radioactive phosphorus in foods. Federation Proc. 24(2), 1965.

RESEARCH AND TECHNOLOGY RESUME	1	2. GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA OA6479	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U U (RPT WRK)	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 097	None

11. TITLE.
(U) Plasma volume expanders in relation to bleeding (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
003500 Clin. Med.; 012900 Physiol.	07 63	NA	OTHER | DA

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

19. GOV'T LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME Headquarters	NAME Walter Reed Army Institute of Research
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RESP. INDIV Tigrett, Col. W.D.	INVESTIGATORS PRINCIPAL Crosby, Col. W.H.
TEL 202-576-3551	ASSOCIATE Shields, Maj. C.E. Eichelberger, J.W.
	TEL 202-576-3365 TYPE [illegible]

21. TECHNOLOGY UTILIZATION	22. COORDINATION
Plasma substitutes	NA

23. KEYWORDS Blood; blood coagulation; blood preservation; blood transfusion; plasma substitutes

24.
(U) Tech Objective - To develop a plasma expander for use when blood is unavailable. To prolong shelf life of whole banked blood.

(U) Approach - Newly developed plasma expanders are being investigated. Effect of newly developed blood preservatives upon survival of stored red blood cells is being
25. evaluated.

(U) Progress (Jul 64 - Jun 65) - Plasma substitutes were developed for use when blood was unavailable and because of the high incidence of hepatitis following transfusion with plasma. Studies in this laboratory showed that the most widely accepted plasma expander (dextran) produced a bleeding abnormality. A similar defect has been observed in patients with an undiagnosed familial bleeding tendency and in iron deficient subjects. The relationship of these abnormalities is being investigated. Newly developed plasma expanders were examined to find a more physiologic blood substitute. Hydroxyethyl starch may be preferable to dextran as a plasma expander.
26. Although hydroxyethyl starch produces a coagulation defect <u>in vitro</u>, a bleeding tendency has not been observed in patients. These problems with expanders have led to examination of additives to whole blood to lengthen shelf life. Addition of adenine has markedly increased storage period. Studies are under way to assure safety of adenine in banked blood.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 097, Plasma volume expanders in relation to bleeding

Investigators.

Principal: Colonel William H. Crosby, MC
Associate: Harvey J. Weiss, M.D.; Capt Richard M. Kaufman, MC; Capt David A. Sears, MC; Mr. James W. Eichelberger; Capt Roger Ewald, MC

Description.

The hemostatic defect caused by infusion of dextran limits the usefulness of this plasma expander. Studies were continued to further characterize this defect, to study related coagulation abnormalities and to evaluate new plasma expanders.

Progress.

The hemostatic defect (prolongation of bleeding time) caused by dextran was first reported from this laboratory in 1954. The coagulation defect was accentuated by infusion of increased concentrations of plasma expander or by administration of dextran with large molecular weight. Further studies showed that dextran interfered with platelet function by coating them and preventing the release of platelet Factor III.

Intravenous infusions of standard dextran or low molecular weight dextran produced a marked transient deficiency of platelet Factor III in 95 percent of normal subjects tested. There was no direct relationship of platelet Factor III deficiency to hemodilution or platelet counts. In vitro studies showed a correction of the dextran induced defect following sonic oscillation. Methods to measure platelet adhesiveness are being developed and evaluated to provide better means of quantifying this defect.

Patients with an undiagnosed mild bleeding disorder were studied and found to have a platelet Factor III deficiency. Administration of cortico steroids temporarily corrected this abnormality in a number of patients and permitted the performance of operative procedures in these patients without abnormal bleeding. Family studies of these patients showed a high incidence of this disorder and in addition there was a frequent occurrence of allergy and hypersensitivity. This combination of a platelet abnormality and an abnormal immune response suggested a similarity between this familial bleeding abnormality and the hemostatic defect observed in patients with hyperglobulinemia and subjects receiving infusions of dextran.

During the performance of coagulation tests to study these abnormalities, control blood was obtained from purportedly normal subjects. Several normal donors were found to have a thrombocytopathy. Further investigation revealed that these donors were iron deficient due to frequent donation of blood. The role of iron-containing enzymes and total platelet iron-content in the production of bleeding abnormalities is being studied.

Methods which prolonged the storage of whole banked blood would increase the quantities of blood available for transfusion and improve logistical support to overseas areas. Recent studies indicated that the addition of adenine to blood preservatives would significantly increase the shelf life of stored blood. The effect of adenine upon the viability of red blood cells is being evaluated to ascertain if this additive should be employed on a large-scale basis.

During the last year hydroxyethyl starch was developed for use as a plasma expander. Preliminary in vitro studies have failed to show the coagulation defect observed with dextran. Infusion of this substance into rabbits showed no antigenic or clotting problems and indicated that this material might be superior to dextran as a clinically useful plasma expander.

Basic studies of platelet production were continued. Methods were developed for quantifying the number of megakaryocytes released from the bone marrow into the peripheral blood, and permitted calculation of platelet production from various sites in the body. Methods were developed to study platelet mass by electron microscopy.

Summary and Conclusions.

Platelet Factor III release is prevented by the coating of platelets with dextran. A similar defect has been observed in various disease states with hyperglobulinemia. A mild bleeding disorder has been studied which is associated with abnormal immune responses and abnormal platelet function. The possible association of this thrombocytopathy with iron deficiency is being studied. A new plasma expander was evaluated which may be superior to dextran for clinical use. Basic studies of platelet formation were undertaken.

Publications.

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RESEARCH AND TECHNOLOGY RESUME

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10a CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 098

10b PRIOR NUMBER/CODE: None

11. TITLE: (U) Chromium complexes of insulin and related compounds (21)

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008300 Inorganic chemistry; 002300 Biochemistry	06 63	05 66	OTHER 1 DA

16 PROCURE. METHOD	17. CONTRACT/GRANT	18 RESOURCES EST.	a PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
B. CONTRACT	a. DATE 06 63 b. NUMBER DA 49-193-MD-2444 c. TYPE A. FPF d. AMOUNT 30,000	PRIOR FY		
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21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Chromium; insulin; chelating agents; mineral metabolism

24. (U) Tech Objective - To study the interaction of trivalent chromium with biological materials, particularly with nutrients which compete for chromium in the GI tract, with carrier substances which bind the element in the blood and with insulin for which chromium is a co-factor. To determine chemical parameters which are essential for biological activity and to synthesize compounds for biological testing.

25. (U) Approach - The influence of pH (degree of olation) and of various potential chelators on the solubility of chromium is determined, using a membrane dialysis technique. Various approaches are under investigation for the purpose of synthesizing new chromium complexes with ligands of biological interest, using aqueous as well as non-aqueous systems.

26. (U) Progress (Jul 64-Jun 65) - Of the substances subjected to in vitro tests to determine their effectiveness in enhancing the action of insulin, solutions of chromium-amino acid complexes have been found quite promising. Since these solutions contain mixtures of olated complexes of different chain length, attempts have been made to synthesize the individual components of the series. To facilitate these studies, a dialysis technique has been developed to fractionate the mixtures. This has also been used in the study of Cr (III) in solutions of the pH range of the intestine and in the presence of substances normally found in the GI tract. The very low solubility in the presence of orthophosphate may explain the poor absorption of chromium from the diet. The solubility was markedly increased by pyrophosphate and amino acids, and decreased by fatty acids.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. FY FUNDS (In thousands)	36.
CY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 098, Chromium complexes of insulin and related compounds

Investigators.
Principal: Carl L. Rollinson, PhD
Walter Mertz, MD

Associate: J. V. Lindsay, BS

Description: It is the aim of this project to gain knowledge as to how the chemical structure of trivalent chromium complexes and chelates influences their biological activity. It has been decided that the research program would include investigations of the following specific interrelated problems: (1) preparation, characterization and evaluation of chromium-insulin complexes; (2) preparation and biological testing of various chromium complexes; (3) preparation of olated chromium complexes of known composition; (4) investigation of formation of chromium complexes from ligands likely to be formed in the digestive process.

Progress: Included in the preparations which have been submitted for testing by the usual procedure have been solutions of chromium-amino acid complexes. Since Dr. Mertz has obtained quite promising results with these solutions, most of our efforts along preparative lines during the period of this report have been directed toward producing complexes of this type of known composition, or solutions containing such complexes.

Aqueous Solutions. Reactions of Cr(III) in aqueous solutions are complicated because of occurrence of hydrolysis and olation. In attempts to define these systems in greater detail, a number of solutions were prepared in such a way that the final concentration of Cr(III) in each solution was .01 M, but with different ratios of chromium to alpha-amino acid (usually methionine). What was hoped for, although the probability of its occurrence is perhaps low, was that the product in each case would be an olated compound containing a specific number of chromium atoms determined by the Cr/amino acid ratio used (i. e., 2:4, two: 3:4, three; 4:4, four; 5:4, five), the four amino acid residues acting as end groups. The more probable alternative is, of course, the formation of a mixture of products of different chain length.

The solutions were prepared as follows. The chromium-amino acid solution (about 80 ml), maintained at 37°C., was neutralized by dropwise addition of Na_2CO_3 solution over a period of about an hour. The

solution was stirred continuously during neutralization. The reaction mixture was finally made up to a volume of 100 ml. All of the solutions were violet. No precipitate formed except in one preparation in which an excess of sodium carbonate was deliberately used.

These solutions were tested in various ways. The spectrum of each solution in the visible region was obtained, and spectra over a period of two to four days were obtained for some of the solutions. Some were lyophilized and the spectrum of the solution made from the lyophilized product was compared with that of the original solution. Some of the solutions were tested by thin-layer chromatography and/or electrophoresis to determine whether mixtures of chromium complexes were present and to separate these from each other.

It was hoped that the spectra could be correlated with composition as determined by TLC or electrophoresis but these methods did not provide the required information since chromium did not move from its initial position. However, this may be regarded as significant and can be interpreted in either of two ways, i. e.: the products of reaction were polynucleate complexes of high molecular weight, or perhaps more precisely large olated ions of low charge mass ratio; or, although the reaction products might initially have been ions of small ionic weight, the equilibria could have been shifted in the process of evaporating the test drops (on the TLC plate or electrophoresis strip) in such a way that large aggregates were formed.

Other valuable information was also obtained from these experiments. The ninhydrin test, which shows the position of uncombined amino acids also gives a positive test with the coordinated amino acid. Even though the chromium did not move, TLC and electrophoresis could be used to purify the complex since these processes move excess amino acid and other substances away from the chromium spot. It appears that changes in composition may occur during lyophilization and re-solution.

The results so far obtained warrant continuation of this work and furthermore the approach can be made more fruitful by use of dialysis as discussed later in this report. By this technique it will be possible to fractionate the reaction mixtures and to define the conditions that determine the ionic weights of the products.

Non-Aqueous Solutions. The difficulty of preparing olated chromium complexes of known composition in aqueous systems arises from the fact that every water molecule is potentially an OH^- ion donor with high probability that a continued process of olation will occur leading to a mixture

of products of different chain length. The alternative to studying such mixtures is to synthesize single specific olated compounds of known composition of the types that would be present in the mixtures. The use of non-aqueous solvents, in which the OH^- concentration can be controlled, appears to be the logical approach.

Some preliminary work along these lines has been accomplished. The starting material is $(Crdmf_3Cl_3)^2$ (dmf = dimethylformamide) which is soluble in N, N-disubstituted amides. The reaction between this complex and the sodium salt of an amino acid should produce chromium amino acid complexes. The sodium salts of amino acids are not very soluble in substituted amides, but a driving force for the reaction exists since NaCl is insoluble. The following reaction therefore occurs:

$$(Crdmf_3Cl_3) + 3\ Na\bar{a}\bar{a} \rightarrow (Cr\bar{a}\bar{a}_3) + 3\ dmf + 3\ NaCl$$

($\bar{a}\bar{a}$ = amino acid ion).

By use of the appropriate Cr/amino acid ratio and addition of the calculated quantity of NaOH, it may be possible also to synthesize specific olated complexes containing known numbers of chromium atoms.

Several unsuccessful attempts have been made to synthesize the tri-methionine complex and the diol by these reactions. The conditions necessary have not yet been established. A quantitative yield of NaCl has not been obtained. The red solutions filtered from the NaCl have been treated by addition of alcohol to precipitate the complex, and by evaporation of most of the solvent in a rotary evaporator, but as yet the products obtained have been sticky and oily, and not suitable for analysis. Further isolation of the chromium-amino acid complexes will be investigated. All the usual precautions were observed to maintain anhydrous conditions, which is difficult because the substituted amides are hygroscopic. In the meantime a glove box made of "Lucite" has been completed and tested. Dry air is provided by an air dryer which makes it possible to attain a dew-point as low as -55°C. In the continuation of the work outlined, manipulations will be performed in the glove box, and with solvents purified by means of a large vacuum fractionating column which we have also recently completed.

Formation of Chromium Compounds in the Digestive Process. During the course of the work described, the necessity of a more fundamental approach became increasingly apparent. Therefore, it was decided to undertake a systematic investigation of the behavior of Cr(III) in solutions containing compounds and ions usually present in the intestine.

It was suggested in the previous report that Cr(III) may be converted in the intestine to complex compounds of types that in vitro tests show to be effective in enhancing the action of insulin.

Results of feeding experiments are significant in this connection. When laboratory animals are fed a diet containing Cr(III), only a small amount of chromium enters the metabolic system, and increasing the chromium content of the food does not increase significantly the quantity absorbed. This at least suggests the possibility that most of the chromium is converted to insoluble compounds.

Potentially the key to the situation is competition for Cr(III) between substances that can form insoluble compounds and those that can form soluble complexes. There are at least two ions present in the intestine that can precipitate Cr(III), namely $PO_4^{\blacksquare}$ and OH^-, although the former might in some circumstances coordinate well enough to minimize olation and thus to some extent prevent precipitation of the hydroxide. Among the substances normally present in the intestine are several known to coordinate strongly (e.g., amino acids, pyrophosphate ion) and others that may have some coordinating tendency (e.g., glucose, fatty acid ions).

The experimental procedure consists of determining the solubility of Cr(III) in solutions of the desired composition and pH. "Solubility" in this context is defined by the method used to measure it. The systems under consideration will contain not only soluble chromium species and solid phase (probably hydrous chromium oxide or a basic phosphate) but also polymeric colloidal ions produced by olation. In the analytical precipitation of $Cr(OH)_3$, these are normally precipitated by heating - an undesirable situation if the conditions of the intestine are to be approximated. The "solubility" actually measured will therefore depend on the pore size of the filter used to separate the solid phase from the solution or of the dialysis membrane in the method to be described.

Of the three procedures that were given consideration (ultrafiltration, centrifuging, dialysis), dialysis was chosen because it appeared to be most suitable for performing a considerable number of experiments simultaneously with a minimum of manipulation. The work described in this report was done with "Visking" tubing, with average pore diameter of 2.4 millimicrons.

The procedure used follows: one end of a length of dialysis tubing was tied off and the other tied around a glass tube inserted into a stopper fitting the vial or the hole in the cap of a screw-cap bottle.

The solution in which the solubility of Cr (III) was to be determined was put into the bottle or vial and also inside the dialyzer tube.

An aliquot of very dilute chromium chloride solution containing Cr-51 was added to the solution outside the dialysis tube. The dialyzers were placed in the rack of an oscillating shaker in a bath kept at 37° C. At intervals, the solution inside the dialysis tubes was sampled and counted on a well-type scintillation counter equipped with a pulse height analyzer set to count with maximum efficiency the gamma peak of Cr-51 (0.32 mev).

Preliminary experiments showed that solubility of Cr (III) in the phosphate solutions in the range 10^{-5} to 10^{-6} M could be expected, with dialysis to equilibrium requiring six to twelve hours. In the experiments described, dialysis was continued to equilibrium. The initial concentration of chromium outside the dialysis tube was chosen to make the equilibrium concentration 10^{-5} M in the absence of precipitation.

These preliminary experiments also showed that if the dialysis tube was immersed in the phosphate-Cr (III) system immediately after the reaction mixture was prepared, extremely erratic results among duplicate samples are obtained. This may be due to a varying rate of olation which in some cases did not provide sufficient time for the particles to grow beyond the minimum size for diffusion through the membrane. To obviate this difficulty, the reaction mixtures were agitated at 37° C for two or three hours before the dialysis tube was inserted.

The results shown in Table I demonstrate the potentialities of the method and permit some very interesting conclusions to be drawn. Solubility of Cr (III) in 0.0018 M Phosphate Solution, pH 7.0 - The solubility in the absence of addition agents is 5.4×10^{-6} M as shown by comparison of the count rate for samples 7-12 with that for samples 1-6 (0.01 M HCl solution of Cr (III)). Since the chromium in the acid solution is not olated, at equilibrium, the count rate of a sample from inside the dialyzer will correspond to the Cr (III) concentration (10^{-5} M) that would be reached if no precipitation were to occur.

Effect of Coordinating Agents - Amino acids, pyrophosphate ion and tartrate are known to coordinate strongly with Cr (III) and this is shown by the results for samples 19-24 (methionine), 37-42 (sodium pyrophosphate) and 61-66 (sodium potassium tartrate), these three increasing the solubility respectively by 18%, 17%, and 39%.

Effect of Glucose - Further work on the effect of concentration will be required. Samples 13-18 show a slight increase in solubility of Cr(III) in the presence of 1.5×10^{-3} M glucose, while a slight decrease is shown by samples 67-72 (10^{-3} M glucose). However, even if this decrease is real, it is overcome by the action of methionine (samples 25-30) which increases the solubility of Cr(III) by about 16% in the presence of 10^{-3} M glucose.

Effect of Soaps - Samples 31-36, 43-48 and 49-54 show that stearate, oleate and palmitate decrease the solubility of Cr(III) to essentially zero. This is regarded as highly significant, since such substances are generally present in the intestine. An obvious next step in the investigation is to study the competition between these compounds and strong coordinators such as amino acids. Furthermore, the results show a considerable difference between the effect of stearate on the one hand, and oleate and palmitate on the other. Further experiments with these substances will be required to determine whether the difference is real and if so what its significance may be.

Effect of Albumin Inside Dialyzer - The results for samples 55-60 show that the final concentration of Cr(III) in the dialyzer was 13.7% greater than the equilibrium concentration for Cr(III) in HCl (samples 1-6), and over 100% greater than the equilibrium concentration in the phosphate solution. This is interpreted to mean that Cr(III) diffusing into the dialyzer combines with the albumin, continually decreasing the concentration of Cr(III) in the solution outside the dialyzer, and thus shifting the equilibrium toward solution of the solid phase.

These results indicate the desirability of an extensive investigation of other substances of high enough molecular weight to be confined inside the dialyzer tube.

A second series (Table II) was limited to a study of the effect of phosphate concentration and pH and a comparison of the diffusibility of Cr(III) in dilute HCl and in water, in which olation will occur.

These samples were run in triplicate and in some cases variations of count rate in a set of three were unaccountably large. Therefore, the results tabulated are not as reliable as one could wish; experiments are in progress to determine the cause of the difficulty.

However, certain trends are made evident by these data. For example, it may be concluded that the phosphate ion is quite effective as a coordinating agent since the solubility of Cr(III) is highest in the solutions containing the greatest phosphate concentration. Competition for Cr(III) by phosphate ion and hydroxide ion is demonstrated by the observation that at any given pH, the solubility is greatest at the highest phosphate concentration generally; a sufficiently high OH^- concentration decreases the solubility at all phosphate concentrations.

The effect of olation even at the low OH^- concentration of water is shown by comparison of the results for the aqueous and 0.01 M HCl solutions of Cr(III). Evidently most of the Cr(III) in the former was in the form of complexes of ionic weight too high to permit diffusion through the membrane. Moreover it is noteworthy that the concentration of Cr(III) is lower than that of even the most dilute phosphate solutions in the pH range corresponding to that of water (say 5.8-6.6); this may be taken as further evidence of complexing of Cr(III) by phosphate with consequent inhibition of olation.

Results of the dialysis experiments described demonstrate the utility of the method with further exploitation planned. Of particular interest is the application of the method to Cr(III) solutions containing mixtures of competing substances. Conceivably enough data may be obtained to make it possible to plan animal feeding experiments involving use of diets devised to maximize or minimize the concentration of soluble Cr(III) in the intestinal contents.

Chemical Analysis of Chromium Compounds - In the previous progress report, a possible alternative to the perchloric acid method for chromium was discussed. This method comprises homogeneous precipitation of chromium hydroxide, centrifuging, decanting the supernatant, washing the precipitate, oxidizing it with sodium peroxide and titrating with ferrous ammonium sulfate with diphenylamine sulfonate as indicator.

Although preliminary results had indicated considerable promise for this method, results of further investigation have been disappointing. While the method is quite rapid, the accuracy is somewhat unsatisfactory, although with careful work the precision is acceptable (2-3 parts per thousand). The results obtained are always low by 5-20 parts per thousand.

Many variants of the procedure were tried to overcome this difficulty but without much success. It was established, however, that urea is preferable to the $NaNO_3$-NaN_3 mixture as a homogeneous precipitant and that the presence of a salt such as sodium nitrate

helps to produce a compact chromium hydroxide precipitate that is not disturbed when the supernatant liquid is decanted.

Although it is not planned to devote much more effort to this method, in further work Cr-51 will be used to determine whether precipitation is complete and whether there is loss of chromium during decanting. There is also the possibility that the chromium hydroxide precipitated from complexes of organic ligands may still contain coordinated organic material which would prevent complete oxidation of the chromium by sodium peroxide.

Aside from analytical considerations, this work provided more evidence supporting the hypothesis that some chelating agents present in the intestine may form chromium complexes from which $Cr(OH)_3$ will not be precipitated at the pH of intestinal contents. Coordination compounds of chromium with such ligands as ethylenediamine are easily converted to $Cr(OH)_3$ by the method described while it is difficult to do this with compounds of more powerful chelating agents such as alpha-amino acids.

Summary and Conclusions: Of the substances subjected to in vitro tests to determine their effectiveness in enhancing the action of insulin, solutions of chromium-amino acid complexes have been found quite promising. Since these solutions undoubtedly contain mixtures of olated complexes of different chain length, attempts have been made, with both aqueous and non-aqueous systems, to synthesize individual compounds of the series of known composition. Although some progress has been made, results are so far inconclusive. To facilitate these studies, a dialysis technique has been developed which may make it possible to fractionate the mixtures. This has also been used in the study of the behavior of Cr(III) in solutions of the pH range of the intestine and in the presence of substances, some of which are chelating agents, normally found in the intestine. By this method it was found that the solubility of Cr(III) in 0.002 M phosphate solutions at pH 7.0 is in the range 10^{-6} to 10^{-5} M. The solubility is markedly increased by pyrophosphate ion and amino acids, and decreased by fatty acid ions. Investigation of several other systems is in progress.

Publications: None

Table I

Solubility of Cr(III) in 0.0018 M Phosphate Solutions, pH 7.00

Samples	Addition Agent[a]	C/m (ave.)	Cr(III) conc. ($\times 10^5$)[b]	Ratio to Conc. Phosphate Alone
7-12	none	5011	0.54	-
67-72	glucose, 10^{-3} M	4577	0.49	0.91
13-18	glucose, 1.5×10^{-3} M	5178	0.57	1.03
19-24	methionine	5935	0.65	1.18
25-30	methionine, glucose	5791	0.62	1.16
37-42	sodium pyro-phosphate	5845	0.63	1.17
61-66	Sodium potassium tartrate	6947	0.75	1.39
31-36	sodium stearate	323	0.04	0.065
43-48	sodium oleate	82	0	0
49-54	sodium palmitate	75	0	0
55-60	none; 1% albumin inside dialyzer	10,551	-	2.11

a - Concentration 10^{-3} M except as noted.

b - Based on equilibrium count of 0.01 M HCl solution of 10^{-5} M Cr(III), samples 1-6 (9289 c/m).

Table II

Effect of pH and Phosphate Concentration on Solubility of Cr(III)
(Solubilities Shown as Molar Conc. x 10^5)

pH	0.02 M	0.002 M	0.0002 M
5.8	.824	.532	.332
6.2	.831	.449	.466
6.6	.850	.418	.405
7.0	.856	.299	.372
7.4	.781	.126	.194
7.8	.649	.095	.157

equilibrium concentration, Cr(III) in 0.01 M HCl, 10^{-5} M

equilibrium concentration, Cr(III) in H_2O, inside dialyzer
0.225×10^{-5} M

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6481	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U U (RPT / WRK)	NA	QR	A. WORK UNIT

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 099

10b PRIOR NUMBER CODE: None

11. TITLE: (U) Kinetics of freeze-drying of biological mixtures (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
010100 Microbiology	07 63	NA	OTHER / DA

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

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21 TECHNOLOGY UTILIZATION	22 COORDINATION
Public Health, Civil Defense	NA

23. KEYWORDS: Biological effectiveness; biological mixtures; biological products; cholera vaccine; freeze-drying; inert carriers; plague vaccine; residual moisture; vaccines.

24. (U) Tech Objective - This work unit is concerned with the interaction of components of biological mixtures during differing cycles of freeze-drying. Of particular interest is the apparent protective effect of inert materials on the maintenance of the biological activity of certain viruses and bacteria.

(U) Approach - The effects of the addition of various inert materials, and of variations in the freezing and drying cycles, on the maintenance of biological activity are being investigated in order to determine the optimum conditions for the preparation of biologicals for human use. In addition, a study is being made of the changes in the freezing and drying cycles which may be required because of differences in total load, size and shape of containers, fill volume, etc.

(U) Progress (Jul 64-Jun 65) - Results indicate that, in general, the optimum conditions for freezing and drying must be determined for each individual biological product. The addition of inert materials is particularly important for the maintenance of the viability of bacteria, and for the maintenance of the biological activity of purified antigens, during the freeze-drying procedure. With the cholera vibrio the most satisfactory survival rate was obtained when the viable organisms were suspended in a medium consisting of 5% sucrose, 10% dextran and 1% monosodium glutamate, while for the EV76 strain of the plague bacillus excellent survival was obtained when the organisms were freeze-dried in a medium containing 5% sucrose, 1% glutamate and 2.5% human serum albumin.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28.	29 OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 099, Kinetics of freeze-drying of biological mixtures

Investigators.
Principal: Joseph P. Lowenthal, Sc.D.
Associate: Patricia L. Altieri, B.S.; Sanford Berman, Ph.D.

Description.

The purpose of this program is to examine the interaction of components of biological mixtures during differing cycles of freeze-drying. Of particular interest is the apparent protective effect of inert materials on the maintenance of the biological activity of certain viruses and bacteria.

Progress.

1. Fill volume.

The effect of fill volume in a single size container on the rate of freezing, length of freeze-drying cycle, and residual moisture content was investigated. A 10 ml vaccine bottle was used as the container and 1, 2 and 5 ml volumes of Minimum Essential Medium (Eagle) were tested. This medium was selected since it is currently being used in the preparation of experimental vaccines.

a. Freezing was accomplished on the cold shelf (-42°C) in the freeze-drying chamber. Product temperature was measured by means of an internal thermocouple. The time required for the test material to reach -30°C (temperature at which freeze-drying is initiated) increased with the fill volume as shown below:

Effect of Fill Volume on Freezing Time

Fill Volume	Freezing Time to -30°C
1 ml	10.5 minutes
2 ml	30.0 "
5 ml	78.5 "

b. Freeze-drying was carried out in 3 separate runs using an automatic cam control to regulate the heat input so that the same conditions were maintained in each instance; i.e., the final shelf temperature of +40°C was reached in 14 to 15 hours. Although the heat input was constant, the time that the material remained at a low temperature, as well as the length of time required to reach +32°C (the product temperature at which the material was removed from the chamber), increased with the fill volume as shown in the following table:

Effect of Fill Volume on Drying Time

Fill Volume	Time Product Remained at -30°C or Lower	Total Drying Time
1 ml	4 hours	17 hours
2 ml	8.5 "	21 "
5 ml	14.5 "	25 "

c. The residual moisture content of the dried material was determined by the official NIH method (drying to constant weight over phosphorus pentoxide at 50 microns pressure), with the following results:

Effect of Fill Volume on Residual Moisture

Fill Volume	% Residual Moisture
1 ml	1.45
2 ml	1.43
5 ml	1.16

It can be seen that the residual moisture content was greater than 1% in all instances. Since a residual moisture content of less than 1% is desirable for killed vaccines, it is apparent that either a longer drying time or extended terminal drying at +32°C must be employed when drying products in Minimum Essential Medium (Eagle).

2. Suspending Medium

a. The effect of the suspending medium on the rate of freezing and residual moisture content of the dried product was investigated. Solutions that are acceptable for use as suspending media for biological products were used in 1, 2 and 5 ml volumes. The media were frozen on the shelf of the freeze-drying chamber at -42°C, and freeze-dried using an automatic cam control (total drying time of 17 hours). Residual moisture was determined by the NIH method. The results obtained with the 1 ml volume, for example, were as follows:

Effect of Suspending Medium on Freezing Time and Residual Moisture

Test Medium	Total Solids Mg/Bottle	Freezing Time to -30°C	% Residual Moisture
1. Distilled Water	0.0	14 min.	0.00
2. Normal Saline	9.0	11 "	0.00
3. BPS*	11.3	16 "	0.88
4. BPS + 2% Dextrose	35.6	12 "	1.97
5. MEM**	13.8	10.5"	1.45
6. MEM + 2% Human Serum Albumin	32.4	18 "	1.85

* Buffered Physiological Saline (0.02 M Phosphate)
** Minimum Essential Medium (Eagle)

The rate at which freezing occurred was dependent not only on the total solids content, but also on the type of material; i.e., protein, sugar, salt, etc. Residual moisture was affected similarly; for example, sugars tend to bind water chemically and require longer drying cycles to remove this water of hydration. For the 2 ml volume, the freezing rates ranged from 21 to 30 minutes and the freeze-dried material (21 hours drying time) ranged from 0.00 to 1.43% in residual moisture. With the 5 ml volume, freezing rates were 55 to 105 minutes and residual moisture contents (total drying time of 25 hours) ranged from 0.00 to 1.15%..

b. The effect of suspending medium and fill volume on the retention of formalin during freeze-drying in a chamber-type dryer was investigated. Previous studies with manifold-type drying demonstrated that formalin concentrated with drying and that the major volatilization (in simple solutions) did not occur until the last stages of drying. Sufficient formalin was added to each medium to bring the final concentration of formalin to 0.2%. The formalin content was determined by chemical assay on both the fluid and the freeze-dried material.

Effect of Suspending Medium on Volatilization of Formalin

Suspending Medium	% Formalin Volatilized		
	1 ml	2 ml	5 ml
1. Distilled Water	100	100	100
2. Normal Saline	99	98	98
3. Buffered Physiological Saline	98	95	97
4. BPS + 2% Dextrose	0	0	10
5. Minimum Essential Medium (Eagle)	42	41	53
6. MEM + 2% Human Serum Albumin	-	-	-

No values were given for Medium #6 because the human serum albumin was denatured by drying in the presence of formalin. The dried product could not be resuspended, but merely swelled and was completely insoluble. The results demonstrated the necessity for neutralization of formalin (by sodium bisulfite) before freeze-drying to prevent denaturation or destruction of antigenic material.

c. The effect of suspending medium on the maintenance of the viability of the cholera vibrio and the EV76 strain of the plague bacillus over freezing and freeze-drying was studied. The addition of inert materials appeared to be important for the maintenance of the viability of bacteria during the freeze-drying procedure, as shown in the following table:

Effect of Addition of Inert Materials on Viability during Freeze-Drying

	Vibrio cholera, Inaba Serotype		EV76 Strain, Plague Bacillus	
Suspending Medium	Normal Saline	S-D-G* Solution	2.5% HSA in Water	Sucrose PG** +2.5% HSA
Live Harvest	10.1	10.3	9.7	9.7
Frozen & Thawed	10.4	10.1	-	-
Dried & Resuspended	4.3	7.7	8.3	9.3

* 5% sucrose, 10% dextran, and 1% sodium glutamate in water.
** 0.01 M potassium phosphate solution containing 7.5% sucrose, 0.07% potassium glutamate.

A loss of 5 to 6 logs in viable count was consistently found, when the cholera vibrio was suspended in saline regardless of the methods used for freezing and freeze-drying. Better recovery was obtained when the vibrios were suspended in the S-D-G medium. With the plague bacillus, the recovery of viable organisms was also improved in the more complex medium.

d. Dimethyl sulfoxide (DMSO) was investigated with regard to its effect on freezing and freeze-drying of a bacterial culture. This chemical has been shown to have a protective effect in the freezing of living material such as spermatozoa, red blood cells, tissues, etc. Vibrio cholera, Inaba serotype, was chosen as the test organism because of its lability and also because viability could be determined with relative ease by means of plate counts.

To determine the effect of DMSO on viability through freezing and freeze-drying, a suspension of vibrios in normal saline was divided into 5 parts, and DMSO was added in increasing amounts. One ml aliquots of each part were frozen and freeze-dried. The log values of the viable counts are shown in the table below:

Effect of DMSO on Viability through Freezing and Freeze-Drying

	% Dimethyl Sulfoxide				
	0	2.5	5.0	10.0	15.0
Live Harvest	9.4	9.5	9.4	9.3	9.3
Frozen & Thawed	8.0	9.1	>9.5	>9.5	>9.5
Dried & Resuspended	<5.0	<4.0	<4.0	<3.0	<2.0

These results, as well as the results of other attempts to incorporate DMSO in the environment, indicated that the chemical had a protective effect during freezing, but did not protect during freeze-drying.

3. Freezing and Freeze-Drying Cycles

Various methods of freezing as well as differing freeze-drying cycles were studied to determine what combination would give the best survival rate. The cholera vibrio suspended in a medium consisting of 5%

sucrose, 10% dextran, and 1% sodium glutamate in water, and the plague bacillus in Sucrose-PG containing 2% human serum albumin, were used as the test materials.

a. The technique of evaporative drying was first investigated. The fluid suspension was chilled to 0°C and vacuum was applied cautiously to the freeze-drying chamber to prevent the suspension from frothing out of the bottle. The fluid then became frozen almost instantaneously. The material was freeze-dried over a 24-hour period with shelf temperature being maintained at -2 to 0°C; the product temperature was 10°C when it was removed from the drying chamber. Plate count showed a loss of 2.5 logs in viable count. The method showed promise, but technical details regarding exact controls must be studied further.

b. Other drying cycles investigated were:

(1) Rapid Drying - Product temperature was increased from -34°C to +20°C over 4½ hours; total time of drying was 5½ hours.

(2) Average Drying - Product temperature was raised to +20°C over a 22-hour period in a stepwise application of heat.

(3) Slow Drying - Product temperature was increased from -40°C to -10°C over a 12-hour period and was held at the latter temperature for an additional 43½ hours.

The results obtained with these cycles are summarized in the table below, in terms of log loss in viability, and indicated that the least loss in viability occurred when the organisms were frozen at -55°C and dried slowly.

Effect of Various Freezing Conditions and Drying Cycles on Viability

	Drying Cycles		
	(1)	(2)	(3)
Freezing Conditions	5½ hrs.	22 hrs.	43½ hrs.
-20°C for 20 hrs., -55°C for 4 hrs.	3.2	4.6	4.1
+5°C for 2 hrs., -55°C for 22 hrs.	3.5	4.4	3.5
-55°C for 24 hrs.	4.6	2.9	1.7

c. The EV76 strain of the plague bacillus was also used to determine the effect of varying the conditions of freeze-drying. A 24-hour growth of the organism on double veal infusion agar was harvested with Sucrose-PG medium containing 2.5% human serum albumin. One ml aliquots were frozen in situ on the cold shelf (-42°C) of the freeze-drying chamber. Exceptions to the preceding are noted in the table.

Effect of Varying the Conditions of Freeze-Drying on Viability

Plague Study	Time of Freezing	Freeze-Drying Time Temperature of Product Below 0° C	Total Time of Drying	Terminal Temp. of Product	Log Loss of Viability
1	3½ hrs.	32½ hrs.	48 hrs.	24°C	1.1
2	3¼ "	31 "	50 "	20°C	0.4
4	6½ "	63 "	68 "	20°C	0.37
					0.55
6	24 "*	23½ "	26 "	22°C	0.89
6	7 days**	85 "	95 "	2°C	0.5

* Product was frozen in a -60°C chamber and remained there until freeze-dried.

** Product was frozen in a -60°C chamber and stored for 7 days before drying.

These preliminary studies suggested that in order to maintain maximum viability this bacterial suspension should be dried at reduced temperatures over an extended period of time and that a terminal temperature of +20°C should not be exceeded.

4. Miscellaneous Studies.

a. It was observed that freeze-dried cholera vaccine, when filled in small quantities (total solids content of 20 mg), picked up residual moisture upon prolonged storage at 4°C. It was believed that the butyl rubber freeze-drying stoppers retained small amounts of moisture, even after freeze-drying, which diffused to the dried product under vacuum on long-term storage. An experiment was set up to test this hypothesis and to determine the optimum methods for processing the stoppers and storing the product in order to minimize this take-up of moisture.

Stoppers were washed and half of the washed stoppers were siliconized. All were autoclaved in 400 ml beakers. Part of each group was set aside and allowed to dry at room temperature overnight; the remainder was heated at 100°C for 5 hours and for 24 hours. Mixture 199 containing 2% human serum albumin was filled into 5 ml bottles, 1 ml per bottle. Stoppers were placed in the raised position on the bottles (156 bottles for each stopper group); the material was frozen and freeze-dried and the bottles were sealed under vacuum. The total solids content of the dried product was 32 mg and the average residual moisture content immediately after freeze-drying was 0.05%. Bottles of each stopper group were then stored at -20, +4, 25, 37, 45 and 56°C. They were tested for residual moisture content after 2, 7, 43, 57, 93 and 162 days after being placed in storage. The results indicated that no less than 24 hours drying at 100°C should be used in the processing of freeze-drying stoppers. Storage at -20°C was satisfactory with all groups, but only the material in bottles sealed with stoppers that had been dried for 24 hours at 100°C held up when stored at +4°C through the test period to date. It is hypothesized that at -20°C the moisture in the stopper was frozen and was

not able to diffuse to the dried product. Testing will be continued during the coming year.

b. Two types of commercially available glass vials, tubing-made vials and blown vials, were compared to determine which would be most satisfactory for freezing and freeze-drying. Approximately 1000 vials, 6 ml capacity, of each type were used to determine the freezing rates of two media. The fill volume was 2 ml and the product temperature was measured by means of an internal thermocouple inserted into a bottle in the center of each group.

Effect of Type of Vial on Freezing Time

	Time Required to Reach -30°C	
Test Medium	Tubing Vial	Blown Vial
Minimum Essential Medium (Eagle)	38.5 min.	46 min.
Buffered Physiological Saline +2% Dextrose	35.0 "	75 "

Because the tubing-made vial has uniform bottom thickness, the transfer of cold to the product was more efficient and the time required for the media to reach -30°C was decreased. It can be assumed that the heat transfer to the product in the tubing-made vials was more uniform, and that the material in these vials probably dried at a faster rate. However, after a 41-hour freeze-drying cycle the physical appearance and residual moisture content of the media in both types of vials were the same. Other working advantages of the tubing-made vial over the blown vial include 1) a larger number of vials can be accommodated in the freeze-drying chamber; i.e., 324 tubing-made vials per tray vs 288 blown vials, and 2) residual moisture determinations by the NIH method can be carried out in the original container using a Mettler Micro Balance, 20 gm capacity, for the weighings because of the lighter weight (7 gms vs 17 gms) of the tubing-made vial.

Summary and Conclusions.

1. Experimental studies have demonstrated a direct relationship between the fill volume and the length of time required for freezing and freeze-drying of biological materials.

2. The suspending medium also influenced the rates of freezing and freeze-drying. Both the type of material (salt, sugar, protein, etc.) and the total solids content of the medium affected the rates at which biological products were frozen and freeze-dried. The suspending medium also influenced the rate at which formalin was volatilized during freeze-drying. The inert materials in the suspending medium were shown to play an important role in the maintenance of the viability of bacteria during freezing and freeze-drying. A medium consisting of 5% sucrose, 10% dextran and 1% monsodium glutamate gave the best results to date for freeze-drying the cholera vibrio, while a medium containing 7.5% sucrose, 0.07% glutamate and 2.5% human serum albumin yielded the best results with the EV76 strain of the plague bacillus.

3. Differing freeze-drying cycles were studied to determine which gave the best survival rate for the cholera vibrio and the plague bacillus. The results indicated that least loss in viability occurred when the organisms were subjected to a slow freeze-drying cycle.

4. Other studies have demonstrated that the more uniform, thinner-walled, tubing-made vial permitted more rapid freezing of the product than did the blown glass vial. It was also shown that, upon storage, dried products took up the residual moisture from the rubber closure, but this was minimized by subjecting the stoppers to 100°C for 24 hours before use.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6482	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 100

10b PRIOR NUMBER CODE: None

11 TITLE: (U) Isopycnic fractional recrystallization of soluble RNA (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002300 Biochemistry; 012700 Physical Chemistry	07 63	NA	OTHER | DA

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

19 GOV'T LAB INSTALLATION ACTIVITY
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20 PERFORMING ORGANIZATION
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INVESTIGATOR PRINCIPAL: Allison, Capt. J.L.
ASSOCIATE: Hartman, Dr. R.E.
TEL: 202-576-3435 TYPE: DA

21 TECHNOLOGY UTILIZATION: Biochemical research

22 COORDINATION: NA

23 KEYWORDS: Biology, molecular; RNA; cesium; centrifugation; research

24 (U) Tech Objective - A combination ot density gradient centrifugation and fractional recrystallization was proposed for isolation ot complex biopolymers. Whereas chemical analysis can distinguish only between nucleic acids containing ribose or desoxyribose, molecules are already known to exist within the ribose group which function as ribosomal or messenger or transfer RNA. Hence the existence of whole families of these special molecules has been demonstrated but their individual separation and specific study is prerequisite to further understanding of protein synthesis, antibiotic action, and the control of growth.

25 (U) Approach - Since there is a specific transfer RNA (soluble RNA; s-RNA) that associates with each of the twenty amino acids, transfer RNA was used in a pilot study to determine the extent to which the material could be separated into subfractions and to check the isolated fractions against the known biological properties. RNA is too dense to study with the usual density gradients, but preliminary experiments had shown that a crystalline derivative would float in cesium chloride gradients.

26 (U) Progress - Practical gradients and s-RNA separations were obtained with a range of centrifuge speeds from 17,250 rpm to 42,040 rpm. The buoyant density was calibrated with refractive index using 2-methyl heptane and cyclohexane between 1.3899 and 1.4238; the optimum separation corresponded to a cesium chloride concentration of 59.7 weight per cent. The tests performed on isolated fractions did not show significant correlation with ability to associate with activated amino acids.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 24 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 100, Isopycnic fractional recrystallization of soluble RNA

Investigators.
Principal: Fred E. Hahn, Ph.D.
Associate: Capt James L. Allison, MSC

Description.

An attempt at separating the individual members of the group of transfer ribonucleic acids.

Progress.

This work unit has been phased out. The preceding Annual Progress Report is considered a final report. Since other methods of separating the transfer RNAs have become available to molecular biology it is doubtful that a continuation of this study would be profitable.

Summary and Conclusions.

This work unit has been phased out.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME	1.	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6483	CSCRD-103

4. DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 101	None

11. TITLE

(U) A correlative approach to acute renal injury (09)

12. SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
012900. Physiology; 002300. Biochemistry; 012600. Pharmacology	07 63	NA	OTHER | DA

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

19 GOV'T LAB INSTALLATION. ACTIVITY	20 PERFORMING ORGANIZATION
NAME Headquarters	NAME Walter Reed Army Institute of Research
ADDRESS U.S. Army Medical Res & Dev Command Washington, D. C. 20315	ADDRESS Washington, D. C. 20012
RESP. INDIV Tigertt, Col. W.D. TEL 202-576-3551	INVESTIGATORS PRINCIPAL Barry, Lt. Col. K.G. ASSOCIATE Rosen, Capt. S. TEL 202-576-3529 TYPE DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Medical Science	NA

23. KEYWORDS

Anuria; kidney; shock; mannitol; renal function; enzymes; renal tubule

24. (U) Tech Objective - Study of acute renal failure requires physiologic, biologic and morphologic correlation.

(U) Approach - Alterations observed by micropuncture, enzyme assay and morphologic techniques are being correlated in a standardized model.

25. (U) Progress (Jul 64 - Jun 65) - In addition to extending studies of tubular flow and pressure, important progress has been made in morphologic and enzymologic studies. To create an enzymological map of the rat kidney, a technique for sampling the various anatomical renal segments has been devised and appropriate enzymes measured. Acid phosphatase (AP), the histological and biochemical marker of the lysosome, an organelle implicated in cellular injury, has been investigated. Results indicate AP is composed of multiple isoenzymes and infer that lysosomal structure is different in the various segments of the rat kidney. Lactic acid dehydrogenase (LDH), an enzyme also associated with tissue necrosis, has been studied. The isoenzyme pattern of LDH also
26. appears characteristic for the different segments of the rat kidney but its distribution is opposite that of AP. Techniques for plastic embedding of relatively large portions of tissue have been developed to facilitate the cutting of thin sections and thereby allow the highest possible resolution by light microscopy. In a combined anatomical and physiologic study of experimental radiation nephritis, these methods revealed fine details of cellular injury implicating both cortical and medullary abnormalities as the basis for the clinically observed polyuria. Previously undescribed collecting duct alterations appear to be the dominant medullary change.

For technical reports, see WRAIR Annual Progress Report, 1Jul 1964-30Jun 1965.

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

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

ANNUAL PROGRESS REPORT

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 101, A correlative approach to acute renal injury

Investigators.
Principal: Lt. Colonel Kevin G. Barry, MC
Associate: Captain Walter H. Glinsmann, MC; Capt Bernard Weiss, MC; Capt Seymour Rosen, MC; and Capt Douglas J. Beach, MSC

Description

This study of experimentally produced renal injuries in animals correlates physiologic, biochemical, and morphologic changes. It encompasses: (A) The development of reproducible models of renal injury. (B) Observation of physiologic alterations, predominantly through the use of micropuncture techniques to clarify pressure and flow relationships in individual nephrons. (C) Biochemical studies of enzyme activity both from the standpoint of maintenance of enzyme integrity as well as translocations in subcellular fractions. (D) Morphologic studies using light and electron microscopy.

Progress

I. Standard models of renal injury in the rat have been developed and include transient total ischemia, hypoxia, and homologous pigment nephropathy, ethylene glycol intoxication, and exsanguination hypotension. These particular models were chosen because of their parallel to various situations of clinical renal injury.

II. Physiologic studies have primarily centered in two areas:

1. In studies utilizing micropuncture techniques, perfusion of individual nephrons during hemorrhage, osmotic diuresis, and ureteral obstruction have been investigated. Tubular perfusion seems to depend on arterial perfusion, glomerular pressure and the osmotic

composition of the glomerular filtrates. Tubular pressure is maintained over a large continuum of blood pressure levels and only ceases below 35 mm. Hg. However, even at the latter levels, hypertonic mannitol will increase tubular perfusion pressure.

2. The model of exsanguination hypotension has been more thoroughly explored. Animals have been found capable to survive periods of hemorrhagic hypotension (at level of 30 mm. Hg.) ranging up to 130 minutes. Physiological data, such as maximal bleed-out volume and reinfusion volume to original pressure have been collected and are being correlated with biochemical and morphological studies.

III. Biochemical studies have centered in three major areas:

1. A simple technique has been develo[illegible] for obtaining representative samples (15-35 mg.) of the various segments of the rat kidney, viz., outer cortex (O. C.), inner cortex (I. C.), outer medulla (O. M.), and inner medulla (I. M., papilla). Recently, a cellular organelle, the lysosome, has been implicated in tissue injury. Acid phosphatase is the traditional histological and biochemical marker of the lysosome and is being extensively investigated. Homogenate and supernatant (37,000 G) from each segment were analyzed for acid phosphatase utilizing para-nitrophenyl-phosphate (PNPP) and beta-glycerophosphate (BGP) as substrates. With PNPP, decreasing activity in both homogenate and supernatant was noted from cortex to papilla: O. C. = 100%; I. C. = 60%; O. M. = 52%; and I. M. = 45%. The % supernatant activity appeared substantially the same in all segments (25%). The optimum pH for maximum enzyme activity was the same for all segments but was reached slightly earlier in the homogenate (5.3) than the supernatant (5.6). With more acid pH (4.4 and 4.7) the papilla homogenate was more active than other segments. The supernatant was more resistant to fluoride inhibition than the homogenate. The I. M. was most resistant, O. M. and I. C. was of intermediate sensitivity and O. C. least resistant. When triton was absent from the incubation media, the I. M. was the only segment where 100% activity was observed. Preliminary data indicated that the Km of the I. M. was different from the other segments which were similar to each other. It is thus suggested

that the acid phosphatase of the kidney is of various types, i. e., isoenzymes. In particular, that of the I. M. seems to be more easily activated and resistant to fluoride inhibition. This latter isoenzyme is probably present to some extent in the O. M. and I. C. and is least in O. C.

Although distribution and % supernatant activity was about the same with BGP, patterns of fluoride resistance appeared to be different and less distinctive. Under varying conditions of molarity and time of incubation, relative enzyme release was lowest in the I. C. and, as with PNPP, was highest in the I. M. Preliminary data indicated that Km of O. C. and I. C. were different; determination of Km of the O. M. and I. M. was difficult because a linear relationship was not found over the range of substrate concentrations used.

These results, particularly with PNPP, suggest the presence of different kinds of acid phosphatase in the kidney. Furthermore, using BGP as substrate, the differential release of acid phosphatase which occurs raises the possibility that the membrane structure of lysosomes may be different in the various renal segments.

Although mannitol is thought to transform lysosomes into large vacuoles presumably more susceptible to trauma, neither the % supernatant or total activity (BGP) of O. C. or I. C. was altered after a 45-minute infusion of 20% mannitol. Earlier results utilizing a relatively mild form of hemorrhagic shock revealed no consistent changes as a result of hemorrhagic hypotension. This was reinvestigated with more drastic hypotension and the studies are as of the present inconclusive. A more extensive series is being contemplated.

Another enzyme associated with tissue necrosis, lactic acid dehydrogenase (LDH) was studied. A distribution nearly opposite that of acid phosphatase occurs with LDH activity: O. C. = 40.9%; I. C. = 32.5%; O. M. = 64.9%; and I. M. = 100%. Investigation of LDH isoenzymes revealed that the M type of LDH dominates in the papilla and the H type comprises the large majority in O. C. Mixtures of both types occur in the I. C. and O. M.

2. Studies of ethylene glycol metabolism suggested that the chief enzymes involved in its catabolism were alcohol dehydrogenase and catalase. The latter are the same enzymes

which oxidize alcohol, and inhibition studies showed that ethanol is a potent inhibitor of ethylene glycol oxidation by both enzyme systems. These results suggest the possible clinical use for ethanol in ethylene glycol intoxication.

3. Enzyme activities in the autolyzing kidney were studied. Autolysis provided a more easily controlled system in which basic information of mechanisms of cellular damage can be studied. Changes in lysosomal enzymes (cathepsin and acid phosphatase) and soluble oxidative enzymes (hexokinase, glucose 6-phosphate, 6-phosphogluconic acid, malic, lactic, and isocitric dehydrogenases) were found. These alterations were prominent only four to eight hours after initiation of the autolysis procedure.

IV. Morphologic studies have developed techniques for embedding large tissue fragments in plastic media. The latter facilitates the cutting of thin sections (1-2 microns). Studies are under way to develop special stains for this material. Where appropriate, all physiological and biochemical experiments have a morphological component. Until recently the latter has been limited to a light microscopic investigation. However, it is hoped that with expansion of existing facilities the large volume of material prepared for electron microscopy can be studied.

V. A combined physiological and anatomical study of experimental radiation nephritis has shown both cortical and medullary abnormalities as the basis for the clinically observed polyuria. Techniques as described in paragraph IV have revealed fine details of radiation injury, i.e., previously undescribed abnormalities of collecting ducts are the most prominent medullary change. Inability to elaborate a concentrated urine correlated with the extent of cortical and medullary damage and the number of days post-radiation. There was also good correlation between days post-radiation and total damage.

Summary and Conclusions

The progress of the Microcorrelative Laboratory has to date provided basic information about physiologic, biochemical and morphologic changes in renal tissue subjected to injury. It is to be expected that this unique multi-disciplinary opportunity to study cellular damage will yield even more fruitful results.

Publications: None

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6484	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	SECURITY WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 102

10b PRIOR NUMBER CODE: None

11. TITLE: (U) Systemic pathologic manifestations of Korean infectious hepatitis (09: KS)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	07 63	NA	OTHER | DA

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

19 GOV'T LAB INSTALLATION: ACTIVITY
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20 PERFORMING ORGANIZATION
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INVESTIGATORS PRINCIPAL ASSOCIATE: Conrad, Maj. M.E.
TEL: 202-576-3557 TYPE: DA

21 TECHNOLOGY UTILIZATION: Hematology

22 COORDINATION: NA

23. KEYWORDS: Hepatitis; viral isolation; kidney; intestine; anemia; pathology

24. (U) Tech Objective - A collaborative effort to isolate the causative agent(s) of infectious hepatitis and delineate the pathophysiology of this disease.

(U) Approach - Isolation of many strains of hepatitis-associated viruses in various laboratories has made identification of the causative agent(s) complex. Supply of aliquots from single specimens obtained from patients with well documented hepatitis
25. permits comparison of results by collaborating laboratories. Lack of a specific test to establish the diagnosis of hepatitis makes it necessary to document carefully the clinical course of each patient and permits scrutiny of extrahepatic involvement.

(U) Progress (Jul 64 - Jun 65) - Specimens from patients with infectious hepatitis were obtained from various geographical areas to maximize the chance of culturing variant viral strains. Aliquots from this virus "bank" were distributed to 17 laboratories to permit comparison of viral agents isolated from these materials. Candidate studies showed hepatitis is a generalized disease with histologic changes in the
26. gastrointestinal, renal and hematologic organs. Physiologic studies showed the commonplace occurrence of malabsorption, anemia and an autoimmune-type hemolytic disorder. Virus-like particles were identified in electron micrographs of jejunum and raised the possibility that hepatitis, similar to poliomyelitis, is a commonplace infection of the gut and only infrequently involves other organs. Documentation of this hypothesis would explain the epidemiology of this disease and provide information of value in the development of a vaccine.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 102, Systemic pathologic manifestations of Korean infectious hepatitis

Investigators.
Principal: Major Marcel E. Conrad, MC; Major Eugene D. Shaw, MC
Associate: Capt Lewis R. Weintraub, MC; M/Sgt Allen A. Young; Roberta Hartman, Ph.D.; Giovanni Astaldi, M.D.

Description.

A collaborative project to isolate and identify the causative agent(s) of infectious hepatitis and delineate the pathophysiology of this disease.

Progress.

During September 1962 and January 1964, field teams were sent from WRAIR to Korea. The diagnosis of infectious hepatitis was established by clinical observation and laboratory studies and was confirmed by examination of liver biopsy specimens from each volunteer. Acute and convalescent specimens of serum, urine and feces were collected from each patient and prepared for transportation, storage and subsequent distribution to virologic laboratories. Distribution of aliquots from single specimens to various laboratories attempting to culture the hepatitis virus permits a better comparison of virologic techniques than attempted isolation of an infectious agent from a variety of sources. Currently, these specimens have been distributed to seventeen virological laboratories that have agreed to work in this collaborative endeavor. In addition, these specimens are being used in experiments to develop a clinically useful laboratory test to identify patients with this disease and in experimental animals to find a susceptible laboratory animal for experimental study.

During the performance of clinical studies to collect infectious materials, the extrahepatic manifestations of infectious hepatitis were investigated. Histologic changes and functional alterations were in the hematological, renal and gastrointestinal organs. Anemia and reticulocytosis were commonplace, and an extracorpuscular hemolytic disorder with megaloblastoid maturation arrest of bone marrow function was observed in a significant number of patients during the acute phase of illness. Renal biopsy specimens showed interstitial edema with a nonspecific glomerulitis and hyaline granular degeneration. Albuminuria and microscopic hematuria were frequent during the late preicteric and early icteric stage of illness. Other clinical tests of renal function were normal. Histologic examination of specimens of gut showed a gastritis during the first week of jaundice and changes in the jejunum and duodenum that persisted for at least four months

after the onset of clinical symptoms. (Studies of gastrointestinal function showed steatorrhea). Specimens of kidney and gut were prepared for examination by electron microscopy. Studies of these specimens are in progress.

Collections of infectious material are being obtained from a variety of geographic areas. These specimens are being placed in storage for use by laboratories attempting to culture the causative agent of hepatitis.

Summary and Conclusions.

Clinical studies of infectious hepatitis were performed in Korea and have been undertaken in a number of geographic areas to provide a source of infectious materials from proven cases of infectious hepatitis. These specimens have been distributed to seventeen laboratories for collaborative viral isolation studies. Hepatitis was shown to be a generalized disease with involvement of the blood, kidney and gut.

Publications.

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Conrad, M. E., Schwartz, F. D., and Young, A. A.: Viral hepatitis: a generalized disease. Amer. J. Gastro. 42: 47, 1964.

Astaldi, G., Conrad, M. E., Ratto, L., and Costa, G.: Misure e particolarità della mucosa digiunale normale ottenuta con biopsia perorale. Minerva Gastroenterologica 10: 121, 1964.

Conrad, M. E., Schwartz, F. C. and Young, A. A.: Infectious hepatitis - a generalized disease. A study of renal, gastrointestinal and hematologic abnormalities. Amer. J. Med. 37: 789, 1964.

Ratto, L., Astaldi, G., Conrad, M., and Costa, G.: Medidas y particularidades de la mucosa yeyunal de la hepatitis epidemica obtenida por biopsia peroral. Estratto da Biochimica e Biologia Sperimentale 3: 195, 1964.

RESEARCH AND TECHNOLOGY RESUME

1.	2 GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6485	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 103	None

11 TITLE
(U) Absorption and loss of radioisotopes by the gut

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
01.100 Radiobiology 012900 Physiology	07 63	NA	OTHER | DA

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

19 GOVT LAB INSTALLATION ACTIVITY	20 PERFORMING ORGANIZATION
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
NA	NA

23 KEYWORDS
Absorption; excretion; iron; intestine; skin; mechanism; radioactive

24 (U) Tech Objective - Study of the mechanisms regulating absorption and excretion of trace elements.

(U) Approach - Using iron^{59} as a model, the factors controlling absorption from the gut and excretion from the body were investigated and controlling mechanisms identified.

25 (U) Progress (Jul 64- Jun 65) - Mechanisms regulating absorption and excretion of trace elements have been poorly understood. Since the body maintains a nearly constant quantity of these substances throughout adult life, a delicate balance must be maintained between absorption and excretion to prevent the disease states caused either by depletion or overloading. The quantity of iron deposited within the duodenal epithelial cell was shown to limit amount of iron salt absorbed from the lumen of the gut. Factors increasing iron utilization by the body depleted intestinal cells and permitted increased absorption of dietary iron. In states of iron-overload these intestinal cells became replete with iron to decrease absorption. Likewise, these cells served as a method for limited but selective loss of the metal when they were
26 sloughed into the lumen of the gut at the end of their lifespan. In hemolytic disorders excessive turnover of intestinal cells permitted simultaneous increases in absorption and excretion of this trace element. Increased absorption of cobalt and manganese in iron depleted animals, but not of other metals, indicated that there was a common but partially selective absorptive pathway. Heme was shown to be absorbed into the body as an intact porphyrin structure and in humans seemed to be a more important source of dietary iron than the metallic salts.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CY++	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 103, Absorption and loss of radioisotopes by the gut

Investigators.
Principal: Colonel William H. Crosby, MC
Associate: Major Marcel E. Conrad, MC; Simeon Pollack, M.D.; Capt Lewis R. Weintraub, MC; Capt Richard M. Kaufman, MC; Capt Stanley Cortell

Description.

Study of the mechanisms regulating the absorption and excretion of radioactive substances.

Progress.

Using radioactive iron^{59} as a model, the factors controlling absorption and excretion were investigated. Studies showed that the quantity of iron absorbed from the lumen of the gut was inversely proportional to the amount of iron sequestered by intestinal epithelial cells. Factors which depleted the mucosal cells of iron, such as an iron deficient diet or an increased body requirement for iron, permitted increased absorption into the intestinal cell and then into the body. Contrariwise, when body stores of iron were excessive, iron was concentrated in intestinal epithelial cells to decrease absorption of dietary iron and permit selective loss of iron from the body in this sloughed epithelium. In animals radioactive iron lost from the body could be accounted for in accumulating fecal collections. Human studies showed that in addition to fecal loss of iron there was appreciable loss from the skin in a limited but selective manner.

The hypothesis that the intestinal content of iron regulated absorption seemed to be challenged by hemolytic disorders in which there was a simultaneous increase in absorption and excretion of iron. To explain this paradox of increased fecal loss with increased absorption and a decreased iron content in the intestinal mucosa we postulated loss from other sources than sloughed intestinal epithelium or increased turnover of these mucosal cells. Both phenomena were shown to be important in rats with acetylphenylhydrazine induced hemolytic disease.

Study of the rate of transfer of iron from the gut lumen to the mucosal cell and from the epithelium into the body showed that both factors were important in regulation of iron absorption and suggested that there was an active transport system in the intestinal mucosa that was largely restricted to the duodenum. Demonstration of competitive inhibition of iron absorption by other metals indicated that this transport system might serve as a common absorptive pathway for a variety of substances. Increased absorption of cobalt and manganese in iron deficient animals, but not of other metals, showed the selectivity of this pathway.

Preliminary studies of the absorption of iron^{59} incorporated into heme showed that porphyrins were absorbed as an intact ring structure. In humans, the absorption of iron salts was significantly depressed when administered with food while heme absorption was not impaired.

Summary and Conclusions.

Factors influencing the absorption and excretion of iron were studied and revealed active and selective transport systems in the intestinal epithelial cells. These studies have provided basic information important in the understanding of intestinal physiology and biochemistry.

Publications.

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Conrad, M. E., Weintraub, L. R. and Crosby, W. H., with the technical assitance of Betty Merrill and Arthur Foy.: The role of the intestine in iron kinetics. J. Clin. Invest. 43: 963, 1964.

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Weintraub, L. R., Conrad, M. E. and Crosby, W. H.: The significance of iron turnover in the control of iron absorption. Blood 24: 19, 1964.

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Kaufman, R. M., Pollack, S., Anderson, Pearl and Crosby, W. H.: Effect of hemolysis on excretion and accumulation of iron in the rat. Amer. J. Physiol. 207: 1041, 1964.

Weintraub, L. R., Demis, D. J., Conrad, M. E. and Crosby, W. H.: Iron excretion by the skin: selective localization of iron^{59} in epithelial cells. Amer. J. Path. 46: 121, 1965.

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6486	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RP / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 104	None

11 TITLE (U) Nature and stability of complexes between biologically important compounds and micronutrients (21)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
008300 Inorganic chem 002300 Biochem 012700 Physical chem	06 64	05 66	OTHER / DA

16 PROCURE. METHOD	17 CONTRACT GRANT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
B. CONTRACT	a. DATE 1 Jun 65 b. NUMBER DA-49-193-MD-2593 c. TYPE A. FPF d. AMOUNT 35,033	PRIOR FY CURRENT FY		

19 GOV'T LAB INSTALLATION ACTIVITY
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Knoblock, Lt Col, E.C.
TEL 301-927-3800 Ext 535 TYPE A

21 TECHNOLOGY UTILIZATION	22 COORDINATION
NA	NA

23. KEYWORDS cation; copper; zinc; cobalt; cadmium; pH; alloxan; salicylates; amino acid; carbonic anhydrase; acid-base equilibrium

24. (U) Tech Objective - To study the nature and stability of interaction between trace metals and organic compounds of biological importance. This interaction is investigated over pH values approximating normal and extremes of the physiological ranges. Anticipated application is further understanding for requirements of acid-base balance.

25 (U) Approach - The techniques of polarography and coulometry have been applied to the study of interactions between metal ions and organic compounds. The effects of incremental pH variations on the formation and stability of complexes are studied in a phosphate medium at ionic strength of blood serum for potential applicability to in vivo interactions.

(U) Progress (Jul 64-Jun 65) - The copper complexes with salicylates, amino acids and vitamin B compounds have been observed to be most active at pH of approximately 7.2. Above pH 6.5 organic compound competes with phosphate for the cation and at a pH greater than 7.5 phosphate dominates. Complexes of zinc and cadmium with salicylate, hydroxyquionones & sulfhydryl compounds were studied. These complexes were not

26 so stable as the corresponding copper complexes & phosphate competed more favorably for the metal ion to pH ranges as low as 6.6. An exception was the complex between carbonic anhydrase & zinc which appeared to be unaffected by the pH of the medium over the range of 6.6 to 7.6. These studies have not confirmed reported complex formation between alloxan & zinc, cobalt, cadmium or nickel. The half-life of the alloxan decomposition was determined as 3.2 minutes in this medium.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 104, Nature and stability of complexes between biologically important compounds and micronutrients

Investigators
Principal: Lt Col Edward C. Knoblock, MSC
William C. Purdy, PhD

Associate: Peter A. Pella, MS

Description: This investigation was initiated with the purpose of furnishing data which describe the varying action of metallic cations as pH changes are brought about in a phosphate medium approximating that of the blood. Cations of known importance as cofactors were chosen for comparison with additional ions of similar chemical activity. Polarographic and coulometric techniques were chosen to add additional detail to the variations of complex formation at differing pH.

Progress: During the first year of this contract work has been started on the study of some of the complexes formed between copper(II), zinc, cadmium, cobalt(II), and nickel(II) and some biologically important compounds. In addition, the behavior of zinc in a phosphate medium varying in pH from 6.6 to 7.6 has been determined with a view to the study of the behavior of the zinc-containing enzyme, carbonic anhydrase. The polarographic characteristics of carbonic anhydrase have been examined and the data indicate that the zinc is retained within the carbonic anhydrase molecule under these pH conditions. Further, a coulometric titration method has been developed for the determination of toxic levels of arsenic in urine. This method involves the isolation of both the plus-3 and plus-5 forms of arsenic, reduction to the plus-3 state with a suitable reducing agent, and titration with generated bromine after a suitable solvent extraction step. Finally initial exploratory work has begun on the polarography of sulfhydryl compounds in the solvent dimethyl sulfoxide (DMSO).

Complex Studies With Copper(II). The polarographic technique has been applied to the measurement of the apparent formation constants of complexes of copper(II). Among the metal-binding agents used were amino acids, vitamin B_6 compounds, and antipyretic drugs. The supporting electrolyte was an orthophosphate buffer of pH 7.4 and ionic strength 0.2 M. Results with fifteen different agents are summarized in table 1.

Also included in this table are the number of electrons involved in the copper reduction and the number of moles of the agent complexing with one mole of the copper(II). The results of this study show that the number of electrons involved in the reduction of copper(II) complexes in a neutral phosphate buffer is not always one. The copper(II)-amino acid complexes all exhibited waves which correspond to two-electron reductions. The other complexes listed in the table exhibited one-electron reduction waves. Hippuric acid did not complex with copper(II), and had no effect on the one-electron reduction wave of copper(II) in phosphate medium. Various other complexes of copper(II) were also studied to ascertain the number of electrons involved in the reductions. Copper(II)-penicillamine and copper(II)-ethyl-enediamine exhibited one-electron reduction waves whereas copper(II)-EDTA exhibited a two-electron reduction. There appears to be no correlation between size and binding strength of the ligands and the number of electrons involved in the reduction of the copper(II) complex.

These studies suggest that the structure of the complex being reduced at the mercury surface may play a part in determining the nature of the reduction product, that is, whether the reduction stops at a copper(I)-phosphate species or proceeds to the metal amalgam.

With Other Cations. The polarographic reductions of cadmium and zinc ions were studied in orthophosphate buffers of ionic strength of 0.2 M over the pH range of 3.00 to 10.40. Cadmium was reversibly reduced over the entire pH range while zinc was irreversibly reduced over this pH range. At a pH of 7.4, zinc was found to have an α-value of 1.0. Above a pH of 5, colloidal suspensions develop in both the cadmium and zinc solutions and it is these colloidal suspensions that are reduced at the DME. Infrared examination of the colloidal suspension is a mixture of the metal monohydrogen and dihydrogen orthophosphate which is reduced.

Table 2 reports the apparent formation constants for complexes formed between zinc, cadmium, nickel(II), and cobalt(II) and several biologically important compounds. At pH 7.4, cysteine, penicillamine, and 2-mercaptoethylamine compete successfully with colloidal metal orthophosphates for the metal ion. The bonding groups in these compounds, the thiol and amino groups, form sufficiently strong bonds to strip the metal from the orthophosphate compound.

Other investigators have described the polarographic behavior of alloxan in other media. Sartori and Liberti studied alloxan by the polarographic technique in 0.1 M potassium nitrate and reported two waves at pH values less than 4. The first wave at 0.18 volt vs. SCE was attributed to the reduction of alloxan to dialuric acid; the second wave appeared at -0.59 volt vs. SCE. Above a pH value of 5, the potential of the first wave changed with time. Hladik reported a shift of the half-wave potential of the first wave through the pH range of 1.8 to 10.7 in Britton-Robinson buffer. Hladik noted three reduction waves before the hydrogen wave at high pH values. The first wave was ascribed to the reduction of anhydrous alloxan while the second wave was thought to be the reduction of parabanic acid formed from alloxan. Ono. Takagi, and Wasu studied alloxan in McIlvaine buffer of pH 3 and reported the half-wave potential of the first wave to be 0.00 volt. Sartori and Liberti reported that alloxanic acid was reduced at -0.59 volt in decimolar potassium nitrate.

In a pH 3 orthophosphate buffer of ionic strength 0.2 M, two waves were observed for alloxan before the hydrogen wave; this was in agreement with the literature. At pH 7.4, three waves for alloxan were observed, in agreement with Hladik. The last wave, at -1.64 volts vs. SCE, was observed to disappear completely with time. This last wave was diffusion controlled and the diffusion current was proportional to the concentration of alloxan in the bulk of the solution. By following the decrease in diffusion current with time, the half-life of alloxan was determined to be 3.2 minutes at 25 $\pm$ 0.2°C. This is a greater value than the 2.2 minutes which was previously reported by spectrophotometric means in a phosphate medium of ionic strength 0.085 M.

Cobalt(II), cadmium, zinc, and nickel(II) do not complex with alloxan in a phosphate buffer of pH 7.4. Lange and Foye prepared and reported 1:1 chelates of copper(II), cobalt(II), and nickel(II) alloxan; however, these appear to be salts. Resnick and Cecil have shown that alloxan does not form a copper chelate and have also shown that the decomposition product of alloxan is alloxanic acid. They reported that alloxan in basic solution with copper(II) does not show any complexation with copper(II) until decomposition to alloxanic acid has been achieved.

Carbonic Anhydrase Studies. Carbonic anhydrase is dependent on zinc for its activity with the zinc atom being held by SH-linkages between histidine and cysteine residues. This positioning of the zinc atom may be essential to enzyme action. Itwas previously reported by Roughton, Booth and others that the activity of the enzyme is pH

dependent. Since the blood is extremely sensitive to slight changes in the pH, an investigation of carbonic anhydrase in an orthophosphate buffer system was initiated to determine if a pH-dependent equilibrium exists in which the zinc is released by the enzyme.

The polarographic behavior of zinc in orthophosphate buffers ranging in pH from 6.60 to 7.60 was first undertaken. These data were then compared with polarographic data for carbonic anhydrase obtained in the same buffer solutions.

The half-wave potential for the zinc reduction does not appear to change within this pH range. The value of the half-wave potential agrees quite well with the value of -1.13 volts vs. SCE for zinc ion in 7.3 M orthophosphoric acid as reported by Meites.

Zinc ion in the phosphate buffer forms a white amorphous precipitate on initial addition. The amount of zinc ion in solution was indeterminable as it changes with time; however, the zinc affords a fairly well-defined wave. The electrode reaction is irreversible and the amount of zinc in solution is controlled by the pH of the buffer.

Two solutions of carbonic anhydrase were prepared for study in the orthophosphate buffers. The concentrations of these solutions were 4.82×10^{-4} and 3.21×10^{-4} M, respectively. Polarograms of these solutions indicated that zinc was indeed present in the enzyme. The amount of the zinc could not be determined because the concentrations of zinc present did not give sufficient current above the residual current for accurate determination of the diffusion current; however, over the pH range of 6.85 to 7.46, the half-wave potential for carbonic anhydrase remained essentially constant. No significant shift of the half-wave potential is apparent as the pH is varied. The diffusion current seems to vary slightly with the pH of the buffers, but due to the scatter of the data, no valid conclusions can be made. The scatter is probably due to experimental error which results from the high sensitivity needed to detect the polarograms.

These data indicate that over this pH range the carbonic anhydrase enzyme is not giving up its zinc to the buffer medium. Evidently, the equilibrium constant for the formation of the zinc-carbonic anhydrase species is greater than that for the zinc orthophosphate species. However, more work is needed to unequivocally determine the pH dependence and to gain some insight into the nature of possible equilibria which compete with the carbonic anhydrase for the zinc ion.

Arsenic Studies. Arsenic determinations are frequently requested of the toxicologist. The common methods of choice are the Marsh or Gutzeit technique. Both methods involve conversion of the arsenic to arsine with the subsequent reaction of the gas to form an arsenic mirror (Marsh) or a colored compound (Gutzeit).

The inherent advantages of coulometric titrations suggested that this method may profitably be applied to the determination of arsenic in urine and tissue. Two problems had to be solved: (1) the conversion of all of the arsenic to the plus-3 oxidation state, which state would be titrated with generated bromine and (2) the removal of arsenic from the other constituents in the sample. The second of these problems was solved first. Plus-3 arsenic can be quantitatively extracted by toluene saturated with hydrogen chloride gas from an aqueous solution which is greater than 10 N in hydrochloric acid. The complete back-extraction into aqueous solutions can be achieved at pH values greater than 3. Plus-5 arsenic is not so extracted, hence the method is capable of determining the amount of plus-3 arsenic in the presence of the higher oxidation state.

Several reducing agents were tested to find the most suitable one for the reduction of plus-5 arsenic to the plus-3 state. Cuprous chloride proved to be the most effective. Working with standard solutions of plus-5 arsenic (0.3434 N in As_2O_5) it was possible to achieve 100% conversion to the plus-3 state and then obtain 100% recoveries from the two extractions.

The following procedure for the coulometric determination of arsenic has been tested with standard solutions of both oxidation states (0.4970 N in As_2O_3), both singly and in admixture. Add 1 ml of the arsenic standard to the reaction flask. Add 1.00 g of cuprous chloride and 10.0 ml of concentrated hydrochloric acid, swirl the mixture, and let it stand for 5 minutes. Empty the contents of the flask into a separatory funnel and wash the flask with 25 ml of toluene (saturated with HCl gas). Extract and remove the HCl (aqueous) layer. Add 20 ml of 1 M sodium acetate to the separatory funnel. Extract and add the acetate layer to a 100-ml volumetric flask. The pH of the acetate extract was found to measure 5.3. Wash down the funnel with distilled water. Repeat with 10 ml of 1 M sodium acetate. To the acetate extract add these washings and 0.55 ml of concentrated sulfuric acid. Cool the solution and dilute to the mark with water. The final solution is approximately 0.1 M in sulfuric acid.

The arsenic in a one-ml aliquot of the diluted sample is then titrated coulometrically with bromine generated at a generation anode from a solution 0.2 N in sodium bromide and 0.1 M in sulfuric acid. The end point of the titration is detected at two biamperometric electrodes between which is impressed a potential difference of 0.2 volt.

Polarography of Sulfhydryl Compounds in Dimethyl Sulfoxide. The solvent DMSO was fractionated at 50°C from calcium oxide at a reduced pressure of 1 to 2 mm through a heated column packed with porcelain saddles. The first 50 to 60 ml of distillate which was collected was discarded; the next fraction (700 to 750 ml) was tested and found satisfactory for the present study.

Tetraethylammonium perchlorate was chosen as the supporting electrolyte. This material was prepared in the following manner. One hundred ml of hot aqueous 1 M tetraethylammonium bromide was slowly added to an equivalent amount of hot aqueous 0.1 M sodium perchlorate. The mixture was stirred constantly during the addition. After cooling the solution in an ice bath, the precipitate was filtered off and was washed with ice water until the wash liquid was free of bromide ion. The precipitate was then recrystallized from water and dried in a vacuum desiccator.

Preliminary studies have begun on the following compounds in the DMSO solvent with tetraethylammonium perchlorate supporting electrolyte: (1) 2-mercaptoethylamine hydrochloride, (2) 2,2'-diaminoethyl disulfide dihydrochloride, (3) cysteine hydrochloride, and (4) the free base of cysteine. At a concentration of 0.002 M, MEA hydrochloride gives three waves, the first of which at -0.22 volt vs. SCE is due to the hydrochloride. The other two waves, at -1.92 and -2.41 volts, respectively, appear to be diffusion controlled and to give a linear relation between concentration and diffusion current. At the present time, the origin of these two waves has not been established.

The polarogram of 2,2'-diaminoethyl disulfide dihydrochloride shows three, and possibly four, waves. The first of these is due to the hydrochloride. In addition, the disulfide exhibits a wave at -1.10 volts and another at -2.40 volts. This latter wave corresponds to the last wave of MEA. There is also a suggestion of a wave at -1.43 volts. None of these waves has been identified.

Cysteine hydrochloride gives waves at -0.22, -1.49, -1.96, and 2.22 volts, respectively. The free base does not have the wave at

-0.22 volt. Considerably more work must be done on these three compounds, and others like them, to determine the nature of the electrode reaction in DMSO.

Summary and Conclusions: Formation constants for fifteen biochemically important compounds were determined for copper with wide divergence being observed between the thiol compounds, amino acids, coenzymes, and salicylates. Electron exchange and ligand values were also determined. The half-time of aloxan in an orthophosphate medium of pH 3.0 was determined to be 3.2 minutes at 25°C. Alloxan did not form a complex with any cations checked. A study of carbonic anhydrase showed that the zinc-protein complex is very stable over the pH range 6.85 - 7.46.

A satisfactory coulometric determination for arsenic was developed which uses toluene (saturated with HCl gas) to extract arsenic(III) from aqueous solution. The arsenic is titrated by coulometrically generated bromine.

The potential for use of dimethyl sulfoxide as a polarographic medium is being investigated. DMSO, with tetraethylammonium perchlorate as electrolyte, shows some unexplained electrode reactions with cysteine and other sulfur containing compounds tested.

Publications: None

Table 1

The Calculated Formation Constants of Copper(II) Complexes

Conc. Cu(II) x 10^4 M	Complexing agent	n	p	K formation
5. 05	Acetylsalicylic acid	1	1	10^3
5. 70	Penicillamine	1	2	2.2×10^{14}
5. 70	Thiosalicylic acid	1	3	2.9×10^{12}
5. 05	S-Acetylsalicylic acid	1	3	2.2×10^{11}
5. 05	Pyridoxine-HCl	1	1	5.9×10^3
5. 05	Niacin	-	-	no complex
5. 05	Pyridoxamine-2HCl	1	1	7.1×10^4
5. 05	Pyridoxal-HCl	1	2	5.0×10^8
5. 05	1-Aminocyclopentane - carboxylic acid	2	2	1.0×10^9
5. 05	Glycine	2	2	1.0×10^9
5. 05	D, L-Leucine	2	2	6.3×10^8
5. 05	Coenzyme A	1	-	complex
5. 00	L(+)-Histidine-HCl	2	2	1.0×10^{13}
5. 00	Hippuric acid	1	-	no complex
5. 00	D, L-Methionine	2	2	1.8×10^9

n is the number of electrons in the reduction of the complex ion.

p is the number of moles of ligand reacting with one mole of the copper(II).

Table 2

Calculated Formation Constants of Metal Complexes

Group I. Cysteine, Penicillamine, 2-Mercaptoethylamine

pH	Ligand	Zn	p	Cd	p
7.4	L-cysteine	complex		7.8×10^{9}	2
7.4	DL-penicillamine	1.3×10^{16}	3	1.2×10^{13}	3
7.4	2-MEA	1.5×10^{6}	2	1.1×10^{9}	2

Group II. Salicylates

pH	Ligand	Zn	p	Cd	p	Ni	p
7.4	salicylic acid	none	-	none	-	none	-
7.4	thiosalicylic acid	complex	-	4.2×10^{9}	2	1.6×10^{19}	3
7.4	aspirin	none	-	none	-	none	-
7.4	thioaspirin	none	-	2.4×10^{4}	1	complex	-

Group III. Hydroxyquinones

pH	Ligand	Zn	p	Cd	p	Ni	p
7.4	Lawsonne	3.1×10^{8}	2	3.2×10^{8}	2	complex	-
7.4	phthiocol	1.4×10^{12}	3	5×10^{11}	3	complex	-

Group IV. Alloxan

pH	Ligand	Zn	Cd	Ni	Co(II)
3.0	alloxan	none	none	none	-
7.4	alloxan	none	none	none	none
7.4	alloxanic acid	none	none	none	none
3.0	alloxan(0.1M KNO_3)	none	none	none	-

a=1.0 for zinc. a=0.9 for nickel.

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6487	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 105

10b. PRIOR NUMBER CODE: None

11. TITLE: (U) Metallic micronutrients and intermediary metabolism (46)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012900 Physiology	07 64	07 66	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT GRANT	18. RESOURCES EST	d. PROFESSIONAL MAN YEARS	e. FUNDS (In thousands)
B. CONTRACT	a. DATE 07 65 b. NUMBER DA 49-193-MD-2595 c. TYPE A. FPF d. AMOUNT 12,973	PRIOR FY CURRENT FY		

19. GOV'T LAB INSTALLATION/ACTIVITY
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20. PERFORMING ORGANIZATION
NAME: Dartmouth Medical School
ADDRESS: Hanover, New Hampshire
INVESTIGATORS PRINCIPAL ASSOCIATE: Schroeder, Dr. H.A.
Mertz, Dr. W.
TEL: 802-254-2331 TYPE: B

21. TECHNOLOGY UTILIZATION: Food technology

22. COORDINATION: NA

23. KEYWORDS: Chromium; vanadium; nickel; germanium; mineral metabolism

24. (U) Tech Objective - To study the effects of chronic nutritional deficiency of certain metallic trace elements in small animals. Particular emphasis is placed on the detection of long-term degenerative processes, like arteriosclerosis, hypertension and diabetes. Under investigation are chromium, vanadium, nickel and germanium, niobium, zirkonium, arsenite, antimony and tin.

25. (U) Approach - Animals are bred and raised in a specially constructed laboratory allowing strict control of trace metal contamination. Diets, deficient in the element under investigation, but supplemented with all other essential dietary factors, are fed the animals during their life span. Chemical analyses, pathological and histological examinations as well as functional tests are carried out on deficient animals and on those receiving the metal involved in normal, excessive and toxic amounts.

26. (U) Progress (Jul 64-Jun 65) - Raising rats under the conditions described leads to the virtual disappearance of glucose removal in many animals, particularly when the chromium deficiency is made more severe by repeated pregnancies. This impairment is accompanied by decreased growth rates and shortened average life span. Chromium deficiency, as well as supplementation with excess lead or cadmium, furthermore results in increased deposition of lipid and increased occurrence of plaques in the aorta. All these symptoms are prevented by chromium supplementation. Experiments are under way to study the effects of long-term supplementation with trace quantities of chromium in chronically hospitalized persons.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC or COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1142) REPLACES DD FORMS 613, 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Investigators.
Principal: Henry A. Schroeder, M.D.
Walter Mertz, M.D.

Description: It is the purpose of this project to study the effects of metallic micronutrients, particularly those for which an essential function has not yet been established, but can be theoretically expected. Since many of those elements are frequently present as contaminants in air, water, and diet, valid information cannot be easily obtained in a conventional laboratory environment. Therefore, the studies are performed in a specially constructed laboratory allowing strict control of trace element contamination. Under these conditions the effects of chromium deficiency and chromium supplementation on various physiological, biochemical and pathological parameters are being measured. Possible biological roles of other elements are studied in life-term experiments.

Progress: During this past year, renovations have approximately doubled the size and output of our animal quarters. One technician has been added and the number of animals under study has increased from 400-800 rats and 600-900 mice.

The research has proceeded according to plan. Life term studies on rats have been initiated with low doses of arsenic, tin, antimony, zirconium, niobium and germanium; on mice with low doses of germanium, antimony, zirconium and niobium. Controls received only traces of these elements. Studies on mice receiving vanadium and tin were completed, those on arsenic are nearly finished. The effect on the absorption of nickel, cadmium and lead on water containing high concentrations of calcium compared to water containing no calcium has been investigated in rats. Analyses are in progress.

Five major tissues are being analyzed for the metals under study by microanalytical chemical methods or by atomic absorption spectrophotometry. Comparative studies on living animals include blood pressure, serum cholesterol, glucose, hemoglobin, urinalyses, growth and mortality rates. Sections for microscopic examination are made on all pathological tissues.

Rats deficient in chromium are being bred for the use of Walter Reed Army Institute of Research and shipped periodically. Furthermore, a diet especially low in chromium has been formulated to ascertain suppression of growth in rats and mice.

Definitive results from the first year of the contract are not yet evident, as was expected in life-term studies. Much data has been obtained, and will be evaluated as each group of animals dies. The effect of 5 ppm chromium(III) in drinking water on growth of rats and mice, previously reported, has been repeated with 2 ppm. Enhancement of growth during the first year of life, especially the first six months, was again demonstrated. No effects on the rates of growth were seen with low intakes of tin, vanadium, arsenic, germanium or niobium.

Chromium-deficient rats have almost uniformly exhibited a low tissue uptake of glucose and have been fully reported. About 25 per cent have exhibited glycosuria after an oral load. Chromium(III) has restored this deficit.

Surveys of the environment of man, including intake in food, concentrations in human and animal tissues, vegetation, soil and water have been completed for arsenic and manganese (2) and are under way for germanium, zirconium and antimony.

Summary and Conclusions: Supplementation with 2 ppm of chromium in the drinking water of rats and mice enhanced growth, whereas that with tin, vanadium, arsenic, germanium or niobium did not. Chromium-deficient rats, raised in this laboratory, exhibited much more severe disturbances of carbohydrate metabolism than did rats from a conventional environment. 25 per cent excreted glucose in the urine of an oral load. This condition was restored toward normal by small, physiological doses of chromium. Definitive results of life-term studies, initiated in this first year of the contract, are not yet evident.

Publications:

1. Mertz, W., Roginski, E. E. and Schroeder, H. A.: Some aspects of glucose metabolism of chromium-deficient rats raised in a strictly controlled environment. J. Nutrition 86:107-112(May) 1965.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6488	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 106

10b. PRIOR NUMBER CODE: None

11. TITLE: (U) Physicochemical Studies of the Biologic Significance of Antiviral Antibody (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 Microbiology	07 64	NA	OTHER / DA

16. PROCURE. METHOD	17. CONTRACT GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
B. CONTRACT	a. DATE 07 65 b. NUMBER DA-49-194-MD-2633 c. TYPE A.FPF d. AMOUNT $29,350	PRIOR FY		
		CURRENT FY		

19. GOV'T LAB INSTALLATION. ACTIVITY
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Artenstein, Malcolm S.
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21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Immunologic factors and preparations; virus diseases; antibodies

24. (U) Tech Objective - This project is aimed at obtaining definitive information of biologic characteristics of various types of antiviral antibody so as to determine their significance in immunity in man.

25. (U) Approach - To study the physicochemical characteristics of antibody of serum and nasal secretions using gel filtration; ultracentrifugation and electrophoresis to determine specific differences and sequential appearance following immunization and infection in animals and man.

26. (U) Progress (Jul 64 - Jun 65) - The data indicates that in man and animals at least 2 components of the immune response occur following immunization or infection, an initial macroglobulin antibody followed by antibody of the 7S gamma G-immunoglobulin type. Certain differences in the persistence of macroglobulin antibody occur and perhaps are related to the status of the viral antigen. In addition, the identification and characterization of viral neutralizing antibody in respiratory secretions as a gamma A-immunoglobulin has been achieved.

For technical reports, see Annual Report to USAMRDC on Contract No. DA-49-193-MD-2633, 1 July 1964 - 30 June 1965.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6489	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U RPT U WRK	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 107

10b PRIOR NUMBER CODE: None

11. TITLE:
(U) Spirometra mansonoides infection and glucose metabolism (09)

12. SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002600 Biology	07 63	NA	OTHER | DA

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

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ASSOCIATE Mertz, Dr. W., Redington, 1/Lt. B.C.
TEL 202-576-3308 TYPE DA

21. TECHNOLOGY UTILIZATION	22 COORDINATION
Medical Sciences	NA

23. KEYWORDS: Spirometra; sparganosis; carbohydrate; metabolism; obesity; cestodes; immune response

24. (U) Tech Objective - Infection in mice with the sparganum of Spirometra mansonoides produces abnormal increases in weight. Investigations were undertaken to gain insight on the role of the parasites in the production of abnormal weight in the host, the factors responsible for fat accumulation and the possibility of establishing a model for studies on adiposity and metabolic abnormalities.

25. (U) Approach - Experiments are set up using recognized immunological and biochemical methods.

26. (U) Progress (Jul 64 - Jun 65) - Some of the biochemical changes in mice infected with Spirometra mansonoides were determined by microtechniques. Infected mice had a post absorptive hyperglycemia, depressed levels of serum alkaline phosphatase, reduced amounts of total protein, essentially normal levels of calcium, chloride, non-protein nitrogen, phosphorus, acid phosphatase, serum glutamic oxalacetic transaminase and a normal proportion of serum components. Previous work showed that an extract of sparganа caused an increase in carbon dioxide production by fat tissue in vitro. A change in methods yielded a worm extract which produced a 700 percent increase in carbon dioxide over controls on reaction with fat tissue. After evidence that the extract acted prior to phosphorylation was obtained, the site of action was determined to be cell membrane. The growth of challenge-infection worms was inhibited by an immune response of the host but not by physiological crowding.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 107, Spirometra mansonoides infection and glucose metabolism

Investigators.
Principal: 1st Lt. Dan Rae Harlow, MSC
Associate: Walter Mertz, M.D., and 1st Lt. Bryce C. Redington, MSC

Description: Recently Mueller reported that mice infected with spargana of Spirometra mansonoides showed accelerated weight gains compared with carefully matched controls. The gain could not be accounted for by the weight of the parasite or associated tissue reactions. The infected animals were simply larger and heavier due to abnormal accumulation of fat. A series of investigations was begun to gain some insight on, a) the possible role which parasites may play in influencing the growth and weight of their hosts; b) the factor(s) responsible for the fat accumulation in the infected animals; and c) the possibiiity of establishing a model for studies on adiposity and metabolic abnormalities.

Progress:

1. Biochemical changes induced in mice by infection with Spirometra mansonoides.

Although there is an abundance of literature dealing with diseases caused by helminth parasites, our knowledge concerning the biochemical changes in infected animals is limited. One of the main difficulties in ascertaining some of the changes brought about by helminthic infections in small experimental animals has been the need for relatively large volumes of serum for clinical biochemical tests. Even when mice were exsanguinated only one or two tests could be performed on individual animals.

A new system of microtechniques was applied to commonly employed clinical biochemical procedures, with minor modifications as appropriate.

Mueller observed that when spargana of Spirometra mansonoides are injected subcutaneously into young mice the host gains weight to an extent which is not accounted for by the weight of the parasites or the associated tissue reaction. Headless fragments produce similar accelerated weight gains, thus indicating that the effect is not uniquely associated with the growing scolex of the worm. No explanation was offered as to the nature of the influence exerted by the worms which might account for the abnormal weight gains in the mice.

In view of the above considerations studies were designed to determine some of the detectable biochemical changes, if any, occurring in mice following infection with this parasite.

Young male and female albino mice, BALBC strain, weighing between 14 and 30 grams were used. Mice were weighed individually at the beginning and at weekly intervals throughout the experiment. The experimental mice were injected subcutaneously with a pre-determined number of scoleces following the techniques of Mueller. The following biochemical tests were used: glucose, glucose tolerance, alkaline and acid phosphatase, total protein, calcium, chloride, non-protein nitrogen, phosphorus, serum glutamic oxalacetic transaminase (SGO-T) and serum electrophoresis. A detailed description of the technique applied, the normal serum values observed, and some of the variables which may alter these values, has been reported elsewhere.

I. Weight Gain

Two experiments were designed to determine whether the spargana induced weight gains in female Swiss Webster mice observed by Mueller, would also take place in male and female BALBC mice used in our laboratory. In both experiments, involving 80 infected mice and 40 uninfected controls, weights were recorded weekly and increases in weight were observed in the infected mice as early as one week following infection. The phenomenon was observed in both sexes regardless of whether the infection consisted of 5 or 10 spargana per animal. The mice in the first experiment were fasted overnight and bled from the tail vein once every 4 weeks to determine the glucose removal rates. Therefore, the weight gains observed on all groups of mice in the first experiment were smaller than those observed in the second experiment which were allowed food and water uninterruptedly.

II. Glucose

An experiment was conducted on 91 mice to determine whether infection with S. mansonoides consistently altered the fasting glucose levels in mice (Table I). Twenty-four mice were given seven worms each (Groups I, III), 27 mice (Group V) were given 15 worms each and all other mice were used as uninfected controls. The difference in glucose levels observed between the mice receiving 15 worms each (Group V) and their uninfected controls was highly significant. Similarly when the values obtained in the infected animals (Groups I, III, V) were compared with those obtained in the uninfected controls (Groups II, IV, VI), the difference was highly significant.

III. Glucose Tolerance

The results of the previous experiment showing higher fasting glucose levels in infected mice suggested the possibility that alterations

TABLE I

FASTING GLUCOSE LEVELS IN MICE 11 TO 18 WEEKS FOLLOWING INFECTION WITH VARYING NUMBERS OF *S. MANSONOIDES*

Exp. Group	No. Mice	Sex	Worms Per Mouse	Weeks After Infection	Glucose MG%	Significance
I	14	♂	7	18	82	P>0.05
II	10	♂	0	-	79	
III	10	♀	7	16	106	P<0.05
IV	10	♀	0	-	72	
V	27	♀	15	11	102	P<0.01
VI	20	♀	0	-	66	
TOTAL	51	♂ and ♀	7 to 15	11 to 18	97	P<0.01
	40	♂ and ♀	0	-	72	

in the carbohydrate metabolism might be brought about in mice by infection with S. mansonoides.

Forty mice were used to compare the glucose removal rates (the percentage of excess glucose removal per minute from the blood) in animals infected with S. mansonoides and their uninfected controls. The glucose removal rates were determined for all mice prior to infection and every 4 weeks up to 16 weeks following infection. As indicated in Table II, no significant differences were observed in the two groups. However, in three of the four times tested (4, 12 and 16 weeks following infection) the infected mice had a higher mean glucose removal rate than their uninfected controls.

IV. Alkaline Phosphatase

In this experiment (Table III) 28 mice were given 7 worms each (Groups I, III),27 mice were given 15 worms each (Group V), and 41 mice were used as uninfected controls. Alkaline phosphatase levels were significantly lower among the infected mice regardless of whether the animals received 7 or 15 worms each and regardless of whether they were tested 11, 16 or 18 weeks following infection.

V. Total Protein

Of the 102 mice studied (Table IV), 29 mice were given 7 worms each (Groups I, III), 27 mice were given 15 worms each (Group V), and all other mice were used as infected controls. Significantly lower total protein values were observed in the infected mice regardless of sex, intensity of infection or whether they were examined 11, 16 or 18 weeks following infection.

VI. Other Biochemical Tests

Several other biochemical tests were performed to determine differences resulting from infection with S. mansonoides. Approximately one-half of the mice were infected with 7 worms each, and the others were used as uninfected controls. The tests are listed in Tables V and VI. No significant differences were observed in the results of these tests.

These studies indicate that mice infected with S. mansonoides had elevated fasting glucose levels, depressed levels of serum alkaline phosphatase and total protein, and essentially normal levels of calcium, chloride, non-protein nitrogen, phosphorus, acid phosphatase, SGO-T and those serum protein components which are determined by cellulose acetate electrophoresis.

The factors which control the blood glucose concentration are so many and so complex that it would be hazardous at the present time to speculate as to the mechanisms by which these changes might be brought

TABLE II

GLUCOSE REMOVAL RATES IN MICE INFECTED WITH S. MANSONOIDES

Exp. No.	No. Mice	Worms Per Mouse	Sex	Glucose Removal Rates at Given Weeks Following Infection*				
				0	4	8	12	16
I	10	7	♂	3.1	3.7	2.5	2.2	3.3
	10	0	♂	3.2	2.0	2.7	1.8	2.4
II	10	7	♀	3.6	2.6	2.3	2.5	3.0
	10	0	♀	3.9	2.6	2.1	2.2	3.4

* The percentage of the injected excess glucose removed from the blood stream per minute

TABLE III

TOTAL PROTEIN LEVELS IN MICE 11 TO 18 WEEKS FOLLOWING INFECTION WITH VARYING NUMBERS OF S. MANSONOIDES

Exp. Group	No. Mice	Sex	Worms Per Mouse	Weeks After Infection	Total Protein GM%	Significance
I	14	♂	7	18	5.8	P=0.01
II	11		0		6.3	
III	15		7	16	5.7	P<0.01
IV	15	♀	0		6.5	
V	27		15	11	5.7	P=0.01
VI	20	♀	0		6.0	
TOTAL	56	♂ and ♀	7 to 15	11 to 18	5.7	P<0.01
	46		0	-	6.3	

about by infection with S. mansonoides. Although it is tempting to attribute the elevated serum glucose levels to a relative or absolute deficiency of insulin, no evidence to this effect was obtained.

The glucose removal rates obtained in infected mice, although usually not significantly different from those of the controls, were definitely not lower as one would encounter in a diabetic curve. The results of this experiment are particularly difficult to interpret since the effect of weight alone on glucose removal rates cannot be separated from the effect of infection per se.

The decrease in alkaline phosphatase values observed in infected mice cannot be fully interpreted. Weimer et al, noticed that serum enzyme levels in rats which had been previously starved, during repletion dropped below normal values. They suggested that this may be due to the fact that during repletion there is a greater synthesis of tissue protein resulting in less release of somatic enzyme, and therefore a drop in serum enzyme levels. It is conceivable that a similar explanation may apply to the mice in these studies which, under the influence of infection with S. mansonoides, were undergoing excessive growth.

The change in total protein concentrations found in infected mice might simply be due to dilution factors. This supposition is supported by the fact that serum electrophoresis analyses indicated that neither albumin nor globulins were primarily responsible for this diminution.

The results indicating no significant differences in the other biochemical tests conducted in infected and uninfected mice were of equally great interest. In particular, it is worth noting that gamma globulins, which usually include most of the circulating antibodies were not increased in infected animals. Likewise, since an appreciable amount of glutamic oxalacetic transaminase escapes into the blood stream following damage to striated muscle, brain, liver and kidneys, the lack of a rise in the serum concentration following infection might suggest that no significant damage to any of these organs resulted from this infection.

In spite of the obvious difficulties of interpreting at this time the mechanisms by which infection with S. mansonoides produced the observed biochemical changes, the fact that consistent changes were indeed found and recognized is noteworthy. The consistency of results obtained in these studies indicates that microtechniques can be used to determine the serum constituents of mice with parasitic infections.

TABLE IV

ALKALINE PHOSPHATASE LEVELS IN MICE
11 TO 18 WEEKS FOLLOWING INFECTION WITH
VARYING NUMBERS OF S. MANSONOIDES

Exp. Group	No. Mice	Sex	Worms Per Mouse	Weeks After Infection	Alk. Phosp. Units	Significance
I	13	♂	7	18	2.2	0.01<P<0.05
II	11		0	-	2.7	
III	15		7	16	1.9	P<0.01
IV	10	♀	0	-	2.7	
V	27		15	11	1.4	P<0.01
VI	20	♀	0	-	2.5	
TOTAL	55	♂ and ♀	7 to 15	11 to 18	1.8	P=0.01
	41		0	-	2.6	

TABLE V

RESULTS OF SIX BIOCHEMICAL TESTS CONDUCTED IN MICE OF BOTH SEXES 16 TO 18 WEEKS AFTER INFECTION WITH *S. MANSONOIDES*

Tests	Serum Required	No. Mice	No. Worms Mouse	Result
Transaminase SGO-T	10	24	7	59 units
		11	0	59 units
Acid Phosphatase	10	23	7	1.1 units
		20	0	1.3 units
Calcium	20	23	7	8.4 mg %
		16	0	8.7 mg %
Chloride	10	24	7	111.1 meq. L
		21	0	115.8 meq. L
NPN	10	23	7	34 mg %
		21	0	37 mg %
Phosphorus	20	23	7	8.9 mg %
		21	0	9.6 mg %

TABLE VI

SERUM PROTEIN CONCENTRATIONS IN MALE AND FEMALE MICE
16 TO 18 WEEKS FOLLOWING INFECTION WITH S. MANSONOIDES

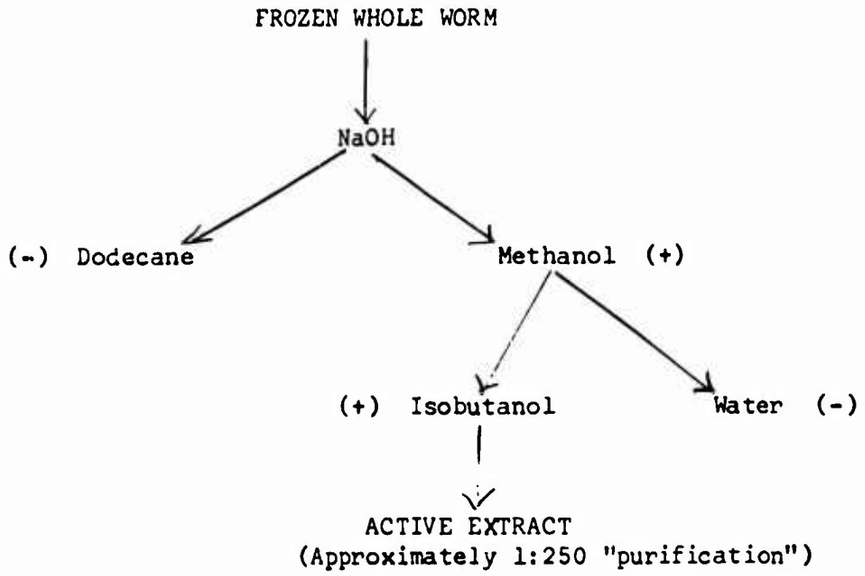
No. of Mice	No. Worms Per Mouse	Mean Concentration (grams)				
		Alb.	Alpha 1	Alpha 2	Beta	Gamma
22	7	2.8	0.4	0.7	1.6	0.4
16	0	3.1	0.6	0.9	1.6	0.6

2. In vitro studies

In cooperation with the Division of Biochemistry studies were initiated to determine the biochemical mechanism of the increased growth rates of the infected mice. As a result of an observation that the extract produced by the previously described method increased in activity when stored at an alkaline pH, a major change in the treatment of the frozen worm was made. The frozen whole worm was placed in NaOH which disintegrated the worm body eliminating the need for homogenizing in a tissue grinder. Thereafter, extractions outlined in the flow chart given in Fig. 1 were performed.

Last year's report showed that rat epididymal fat tissue in vitro (using the method of Renold et al, 1960, as modified by Mertz) exhibited increased CO_2 production in the presence of S. mansonoides extract. These results were confirmed and extended. A 700 percent increase in CO_2 production over controls was demonstrated when 5.0 mg. of extract was used with 250 mg. of fat tissue. This increased CO_2 production is only one of several possible sites of action for the extract. Figure 2 is a basic diagram illustrating possible sites of action.

Lipid production was also determined using the in vitro method of Leonards et al, 1962, as modified by Mertz. A 350 percent increase of lipogenesis was observed with 5.0 mg. of extract (Fig. 2). As the enzyme systems for lipids (4) and CO_2 (5) are entirely different, it is unlikely that the extract could have operated at both sites. Hence, the extract must likely have its effect prior to or during phosphorylation (2). In order to test this site, a sugar that is not phosphorylated was used. Using the method of Resnick and Hechter, 1957, as modified by Mertz, it was established that the entry of galactose into rat epididymal fat cells in vitro was enhanced by the presence of



(+) Activity present

(-) Activity not present

Fig. 1

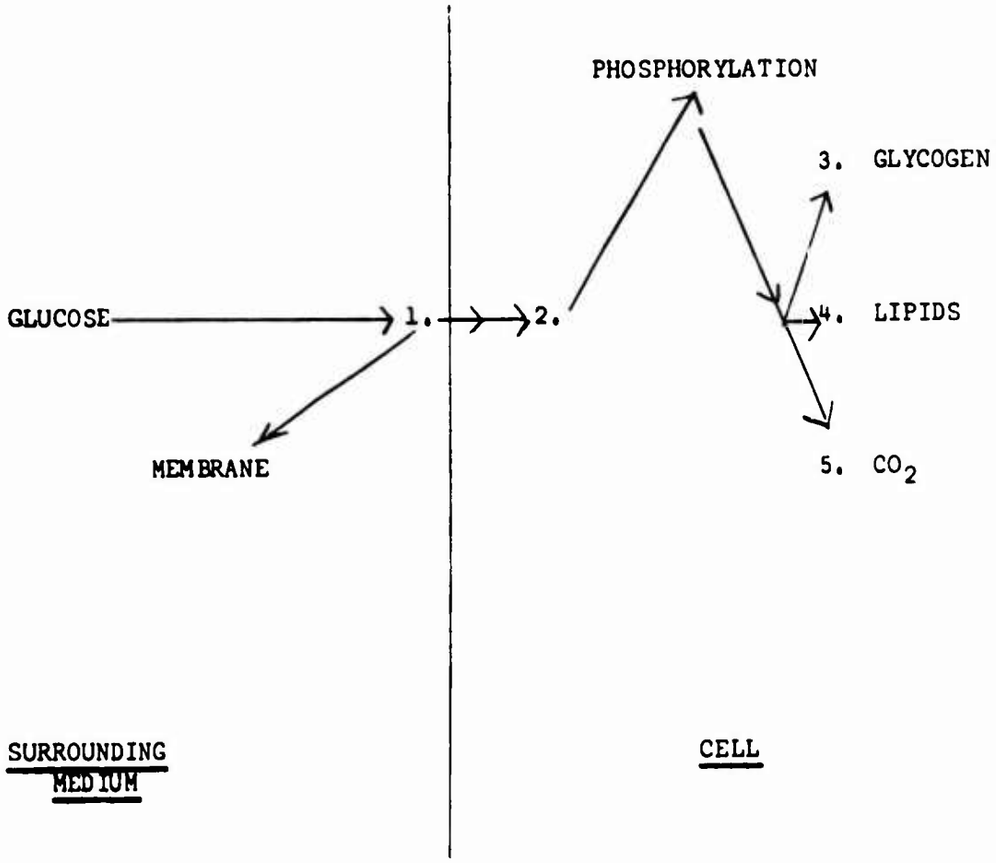

PHOSPHORYLATION
3. GLYCOGEN
GLUCOSE
1.
2.
4. LIPIDS
MEMBRANE
5. CO_2
SURROUNDING MEDIUM
CELL

Fig. 2

the extract. This removed phosphorylation as a possible site of action.

The only remaining cellular site of action was the cell membrane itself. Therefore, the extract must have affected the cell membrane in vitro so that the entry of glucose and galactose was enhanced. It is significant to note that the cell membrane is also the site of action for insulin and that insulin also enhances the entry of glucose and galactose.

As it would be possible to explain the foregoing on the basis of a damaged cell membrane, rat epididymal fat tissue was incubated with extract and inulin, a polysaccharide which does not penetrate intact cell membranes. Since the presence of the extract did not affect inulin penetration, a non-specific membrane damage can be ruled out as a mode of action.

In assaying for in vivo activity using fasted rats, extracts were injected or administered by mouth to determine if a depression in fasting blood glucose levels occurred. Rats tested in this way did not exhibit sufficient depression in glucose levels to be considered significant. Thus the in vivo aspect still is not clearly defined.

3. Acquired resistance in mice to infection with the plerocercoid larvae of the cestode, Spirometra mansonoides.

Attempts were made to determine whether or not mice develop an immunity to infection with S. mansonoides. Mice of similar age were divided into three groups and those in two groups were inoculated subcutaneously with 10 worm scoleces each. Eighteen weeks later, mice in one of the infected groups and the uninfected controls were each challenged with seven additional worms. Seven weeks after challenge all mice in the three groups were sacrificed and the worms were recovered and weighed. Previous experiments had shown that the growth of the parasites was determined most accurately by measuring their wet weight. A statistically significant difference was found between the average weight of the worms resulting from the challenge infection from superinfected mice as compared with those obtained from previously uninfected controls. There was no significant difference observed in the mean weights of worms resulting from the primary infection. Two similar experiments gave corresponding results. The results are summarized in Table VII.

To determine whether or not the inhibition of growth in the worms of the challenging infection was due to acquired resistance or to physiological crowding, another series of experiments was designed in which mice of the same age were divided into five equal groups and given 5, 10, 20, 40, and 80 scoleces each, respectively. At necropsy, seven weeks after infection the mean weight of worms recovered from

TABLE VII

Reduction in wet weight of challenge Spirometra mansonoides 7 and 8 weeks after injection

Group No.	No. Mice	Worms in Orig. Infection	Worms in Challenge Infection*	Wet Weights	
				Orig. Infection	Challenging Infection
A	13	10	7	32 mg	13 mg
B	12	10	0	36	---
C	13	10	7	---	17 mg
A	10	20	7	24 mg	12 mg
B	12	20	0	25 mg	---
C	15	0	7	---	16 mg
A	15	10	7	23 mg	10 mg
B	14	10	0	26 mg	---
C	15	0	7	---	13 mg

* Mice in Experiment 1 sacrificed 7 weeks after challenge and in Experiment 2 and 3, 8 weeks after challenge.

mice in all groups was essentially the same (Table VIII). This indicates that the number of parasites present was not responsible for the inhibition of growth observed in the first series of experiments.

TABLE VIII

Effect of physiological crowding on the wet weight of Spirometra mansonoides in mice

Group No.	No. Mice	No. Worms/ mouse	Worm wet weight*
A	5	5	18 mg
B	5	10	20 mg
C	5	20	16 mg
D	5	40	15 mg
E	5	80	17 mg
A	5	5	17 mg
B	5	10	16 mg
C	5	20	16 mg
D	5	40	17 mg
E	5	80	18 mg
A	5	5	16 mg
B	5	10	19 mg
C	5	20	17 mg
D	5	40	15 mg
E	5	80	18 mg

* Experiment No. 1, Necropsy 7 weeks after exposure.
* Experiment No. 2, Necropsy 8 weeks after exposure.
* Experiment No. 3, Necropsy 8 weeks after exposure.

Summary and Conclusions.

1. Some of the biochemical changes in mice infected with Spirometra mansonoides were determined by microtechniques. Mice had a post absorptive hyperglycemia, depressed levels of serum alkaline phosphatase, reduced amounts of total protein, essentially normal levels of calcium, chloride, non-protein nitrogen, phosphorus, acid phosphatase, serum glutamic oxalacetic transaminase and a normal proportion of serum components. The consistency of results indicates that microtechniques may be used to obtain estimations of the serum constituents in mice with parasitic infections.

2. A change in methods yielded a worm extract which produced a 700 percent increase in CO_2 over controls on reaction with fat tissue. After evidence that the extract acted prior to phosphorylation was obtained it was determined that the site of action was the cell membranes.

3. An acquired resistance to S. mansonoides was demonstrated in mice.

Publications.

Harlow, Dan R., W. Mertz, and J. F. Mueller. 1964. Effects of Spirometra mansonoides infection on carbohydrate metabolism. II. An insulin-like activity from the sparganum. J. Parasit. 50 (Suppl.):55.

Sadun, E. H., Williams, J. S., Meroney, F. C., and Mueller, J. F. Effects of Spirometra mansonoides infection on carbohydrate metabolism. I. Glucose tolerance rates in mice. J. Parasitol. 50 (Suppl.): 54, 1964.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6490	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10a. CURRENT NUMBER/CODE	10b. PRIOR NUMBER/CODE
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11. TITLE: (U) Cytogenetic and metabolic determinants in the evolution of cell population following injury (21)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012900 Physiology, 014100 Radiobiology, 016200 Stress Physiology	11 64	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
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		CURRENT FY		

19. GOV'T LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME Headquarters ADDRESS U.S. Army Medical Res & Dev Command Washington, D. C. 20315 RESP. INDIV. Tigertt, Col. W.D. TEL. 202-576-3551	NAME Walter Reed Army Institute of Research ADDRESS Washington, D. C. 20012 INVESTIGATORS PRINCIPAL ASSOCIATE Glinos, Dr. A.D. Hargrove, D.D., SP4, Burnette, D.W., PFC TEL 202-576-5284 TYPE DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
Clinical medicine	NA

23. KEYWORDS: Genetics; chromosomes; chromosome abnormalities; cellular injury; tissue culture; stress; injury; physiology, cellular

24. (U) Tech Objective - To investigate alterations of structure and function of the genetic apparatus relative to control of cellular metabolism in cell populations surviving injury. Such alterations modify subsequent responses of the organism, favorably, as for example in the immune response following infection or unfavorably as in the rapid ageing or tumor formation following irradiation. The enhancement of favorable and suppression of unfavorable responses in survivors of injury are the ultimate goals of this work.

(U) Approach - To maximize experimental control, L-strain mouse fibroblasts grown in a biphasic logarithmic-stationary suspension culture system are used. The immediate response of this system to injury was shown to approximate closely the response of body tissues. In order to obtain a firm baseline for the structural analysis of the genome of cell populations in this system, the development of a new, high resolution method of quantitative karyotyping was undertaken.

(U) Progress - The chromosomes of the L-strain mouse fibroblasts were analyzed and compared with respect to the frequency distribution of their number, type and size. It was found that the number of chromosomes with interstitial centromeres is a linear function of the total number of chromosomes per cell and that the more central the location of the centromere the greater the relative number and size of these chromosomes. The mathematical formulation of these relationships provides not only a firm baseline for karyotype analysis of cell populations surviving injury but also a deep insight into the processes responsible for chromosomal structural transformation.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 108, Cytogenetic and metabolic determinants in the evolution of cell populations following injury

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: Don D. Hargrove, SP4; Donald W. Burnette, PFC ; Stephen J. Huber, Pvt.

Description:

Although the mechanisms controlling cell growth and regeneration following injury have been under intensive investigation, not enough attention has been given to possible alterations in the surviving and regenerating cells. Yet such alterations, when permanent, modify subsequent responses of the organism favorably, as for example in the immune response following infection, or unfavorably as in the rapid ageing or tumor formation following radiation injury. Permanent alterations necessarily involve the genetic apparatus and in order to be able eventually to enhance favorable and suppress unfavorable responses, methods and criteria must be developed for the structural and functional analysis of the genome of cell populations surviving injury.

In order to maximize experimental control, accuracy of quantitation and analytical resolution, mammalian cell populations growing in vitro rather than tissues in situ, are used. Under the proper cultural conditions these populations have been previously shown to respond to injury in a manner paralleling closely the response of body tissues. Thus, for example, cell loss is followed by activation of cellular biosynthesis, cell growth and regeneration, radiation injury is followed by a limited number of divisions with subsequent loss of the reproductive ability of the cells, and anoxia or starvation is followed by accumulation of lactate similar to the one seen in shock.

Accordingly, the establishment of the proper criteria for detailed analysis of the chromosomal complement of these cell populations has been defined as the first objective of this study.

Progress:

The cell populations used were all clonal derivatives of the mouse fibroblastic strain L-929 established through previous single cell isolations. Thus, clones WRL-5, WRL-7, and WRL-8 were isolated on October 16, 1962 and carried as monolayer cultures in T-60 flasks. Clone WRL-10A was isolated October 23, 1962 and carried as a monolayer

culture until June 14, 1963, at which time it was placed in suspension and continued as a suspension culture until the present.

Standard procedures were used in carrying out chromosome counts. The cells of each population were grouped into the following classes on the basis of their chromosome number: <30, 30-39, 40-49, 50-59, 60-69, 70-79, and >80 chromosomes/cell. The class which contained the highest percentage of cells was considered to represent the stemline or model range characteristic of the population.

Standard karyotyping procedures, on the other hand, were considered inadequate for the purposes of this work and accordingly the following new method was developed: From each cell population a number of typical cells was selected representing the classes with chromosome numbers below 40, 40-49, 50-59 and 80 and over. Microphotographs of these cells were made and idiograms prepared by arranging the chromosomes according to centromere position and size. Four types of chromosomes were identified by centromere position as: telocentric, with terminal centromere; subtelocentric, arm ratio of 2:1 or greater; submetacentric, arm ratio less than 2:1; and metacentric, arms of equal length. Dicentrics and any other chromosomes not fitting into one of these four types were classified under the general heading abnormal. The length of the longest telocentric chromosome of each cell was measured using an ocular micrometer, calibrated with a stage micrometer. The length of each chromosome in the cell was then measured from the idiogram and using the longest telocentric as a reference standard (S), the chromosomes were grouped into 10 size groups on the basis of their length relative to the standard. Four groups: A, B, C, and D of relatively short chromosomes with length ratios: A/S = 1.0; B/S = 0.8; C/S = 0.6; D/S = 0.4; and six groups: Z, Y, X, W, V, and U of relatively long chromosomes with length ratios: Z/S = 1.2; Y/S = 1.4; X/S = 1.6; W/S = 1.8; V/S = 2.0; and U/S >2.0, were formed. In each size group, the ratio as stated represented the upper limit of length for that group. Each chromosome was thus classified both according to its centromere position and to its size relative to the longest telocentric. The percentage of the chromosomes of each type and size was then calculated and a quantitative karyotype table constructed for each cell by arranging the values thus obtained in the manner shown in Table II.

Both the chromosome counts and the karyotype analysis showed no differences among the six populations analyzed. Therefore, data on the frequency distributions of the chromosomes classified by centromere position and relative size, in hypodiploid (<40 chromosomes), hyperdiploid (40-49 chromosomes), stemline or modal range (50-59 chromosomes), and high-ploid (>80 chromosomes) cells from all six populations were compared as shown in Tables I and II. Table I shows that the percentage of the different types of chromosomes did not vary significantly among four classes of cells with widely different total

chromosome numbers. The constancy of these percentages indicates that the number of chromosomes with interstitial centromeres is a linear function of the total number of chromosomes per cell. The mean values of these percentages (bottom line of Table I) increase by approximately a factor of 2 with the sequence: subtelocentric-submetacentric-metacentric indicating that the relative number of these chromosomes increases as the centromere is located more centrally.

The distribution of relative size of the various types of chromosomes in cells with different chromosome numbers was virtually identical so that, the quantitative karyotype tables from all twenty-four cells analyzed may be combined as shown in Table II. The relative lengths shown in this table are expressed as fractions of the length of the longest telocentric chromosome which was found to have a value of 4.52 ± 0.67 micra. Except for its expected greater variation among individual cells, this value is very close to the value of 4.80 micra reported for the longest telocentric chromosome of normal mouse cells. If the relative lengths of normal mouse cell chromosomes are determined on the basis of the idiograms reported by Levan and Hauschka (J. Natl. Canc. Inst. 21:437, 1958) and the size distributions thus obtained corrected for the presence of approximately 44 per cent of nontelocentric chromosomes in the L cells, the following distribution is obtained A = 9.8 per cent; B = 15.4 per cent; C = 23.9 per cent; D = 7.0 per cent. This is in reasonably good agreement with the relative size distribution of the telocentric chromosomes of the L cells shown in Table II. It might therefore be concluded that both with respect to absolute size and to relative size distribution, the telocentric chromosomes of the L cells, constituting approximately 56 per cent of the total complement, do not show any marked deviation from the norm. This is in sharp contrast to the chromosomes with interstitial centromeres. From the figures in Table II it can be calculated that the sum of subtelocentric, submetacentric and metacentric chromosomes, constitutes 42.5 per cent of the total complement and that 88 per cent of these chromosomes are longer than the longest telocentric. In addition, it can be seen from the percentages of the relative size groups which are outlined with heavy lines in Table II that the highest per cent values of the subtelocentric chromosomes are found in the relative size groups of 1.2 to 1.4, while the highest percentages of the submetacentric and metacentric chromosomes are in the 1.4 to 1.6 and 1.4 to 1.8 relative size groups, respectively. This indicates a direct relation between chromosome length and centromere location, the length tending to increase the more centrally the centromere is located.

The karyotype shown in Table II allows us to gain a deep insight into the mechanisms responsible for chromosomal structural transformations. From the frequency distributions previously discussed it may be concluded that the following explanations cannot account for the origin of chromosomes with interstitial centromeres in L cells

and which are absent from mouse tissue cells in situ: pericentric inversion because then the large majority of such chromosomes should be equal to or smaller than the longest telocentric chromosomes (c.f. Table II) and isochromosome formation because it cannot account for the presence of a constant fraction of submetacentric and subtelocentric chromosomes (c.f. Table I). The occurrence of these processes in populations of L cells must therefore be limited and the most probable explanation for the appearance of chromosomes with interstitial centromeres is whole arm translocation.

Whole arm translocation is by far the most common type of structural rearrangement in phylogenesis where it may lead to variation in chromosome number while the number of chromosome arms is nearly constant, i.e. the Robertsonian type of transformation. While this type of transformation has been demonstrated during the evolution of mammalian cell populations in vitro, the findings summarized in Table I indicate that this is not the mechanism responsible for chromosome number variation in L strain cells. The table shows that chromosomes with interstitial centromeres represent a constant fraction of the total number of chromosomes per cell. This would indicate that frequency of whole arm translocation, is determined by chromosome number and that variations in the latter precede the former. While the molecular mechanisms involved are as yet unknown, one possible way that chromosome number could influence frequency of translocation is through spatial interrelations similar to the ones known to operate in radiation-induced aberrations.

Besides chromosome number, the results of the present study would indicate that chromosome size is also of importance in determining frequency of whole arm translocation. From Table II it can be seen that whole arm translocations among the larger telocentric chromosomes of Groups A and B would yield metacentric chromosomes represented by sums W + V + U and X + Y, respectively. Each of these two sums contains approximately 10 per cent of the total chromosomal complement. On the other hand translocations among the smaller telocentrics of groups C and D would yield metacentric chromosomes represented by the sums A + Z and B + C. These two sums contain, respectively, 4.4 and 0.1 per cent of the total chromosomal complement. The existence of a small percentage of chromosomes with a relative length greater than 2 (Group U) would indicate translocations involving all the members of a small initial population of telocentric chromosomes longer than A. Similar considerations apply also to the submetacentric and subtelocentric chromosomes and indicate that the majority of these chromosomes would originate from translocations among telocentric chromosomes of Group A on one hand, one of the other Groups B, C, or D on the other. The relationship between chromosome size and centromere location shown in Table II would thus be explained. In addition, the fact that the fractions of the total chromosomal complement represented by submetacentric and subtelocentric chromosomes are approximately 50 and 25

per cent of the fraction represented by the metacentrics would indicate a rapidly decreasing frequency of whole arm translocation as the sizes of the translocating chromosomes become more unequal.

In conclusion, our new method of quantitative karyotyping illustrated in Table II provides a firm baseline for characterizing normal L cell populations, for detecting possible chromosomal structural changes in populations surviving injury and for visualizing the processes involved in such changes.

Summary and Conclusions:

The objective of this project is to investigate alterations of structure and function of the genetic apparatus relative to the control of cellular metabolism in cell populations surviving injury. Such alterations modify subsequent responses of the organism, favorably, as for example in the immune response following infection, or unfavorably as in the rapid ageing or tumor formation following irradiation. The enhancement of favorable and suppression of unfavorable responses in survivors of injury are the ultimate goal of this work.

To maximize experimental control, L-strain mouse fibroblasts grown in an in vitro suspension culture system are used. The immediate response of this system to injury was shown to approximate closely the response of body tissues. In order to obtain a firm baseline for the structural analysis of the genome of cell populations in this system, its chromosomes were analyzed and compared with respect to the frequency distribution of their number, type and size. It was found that the number of chromosomes with interstitial centromeres is a linear function of the total number of chromosomes per cell and that the more central the location of the centromere the greater the relative number and size of these chromosomes. The mathematical formulation of these relationships provides not only a firm baseline for karyotype analysis of cell populations surviving injury but also a deep insight into the processes responsible for chromosomal structural transformation.

TABLE I

FREQUENCY DISTRIBUTION OF CHROMOSOMES ACCORDING TO CENTROMERE POSITION IN CELLS WITH DIFFERENT CHROMOSOME NUMBERS

Chromosome Number	No. Cells Analyzed	Telocentric (♂)	Subtelocentric (♂)	Submetacentric (♂)	Metacentric (♂)	Abnormal[a] (♂)
< 40	7	57.6 ± 2.15[b]	3.7 ± 1.04	14.8 ± 1.91	22.9 ± 2.27	[illegible]
40-49	5	54.2 ± 2.62	6.1 ± 1.65	12.2 ± 2.86	26.5 ± 1.97	1.0 ± 0.24
50-59	6	57.2 ± 1.04	6.2 ± 0.65	10.2 ± 1.64	24.5 ± 1.62	1.8 ± [illegible]
> 80	6	55.2 ± 0.45	4.6 ± 0.44	12.3 ± 1.40	25.8 ± 1.89	1.6 ± 1.76
Total:	24	Mean: 56.2 ± [illegible]	5.1 ± 0.51	12.6 ± 0.97	24.8 ± [illegible]	1.3 ± [illegible]

[a]Includes all chromosomes other than the four types designated.
[b]Standard error.

TABLE II

SIZE DISTRIBUTION OF CHROMOSOMES WITH DIFFERENT CENTROMERE POSITIONS

Relative Length[a]	Telocentric (%)	Subtelocentric (%)	Submetacentric (%)	Metacentric (%)	Abnormal[b] (%)
U > 2.0		0.6[d]	1.3	1.7	0.2
V = 2.0[c]		0.3	1.1	2.9	
W = 1.8		0.2	1.3	5.4	0.3
X = 1.6		0.2	2.4	5.1	0.1
Y = 1.4		1.2	3.1	5.2	0.1
Z = 1.2		1.6	1.5	3.6	0.2
A = 1.0 = 4.52 μ ± 0.67[e]	9.1	0.5	0.7	0.8	0.2
B = 0.8	18.5	0.3	1.1	0.1	0.2
C = 0.6	19.2	0.2	0.1		
D = 0.4	9.4				
Total	56.2 ± 0.89[e]	5.1 ± 0.51	12.6 ± 0.97	24.8 ± 0.98	1.3 ± 0.32

[a]Fraction of longest telocentric (A).
[b]Includes all chromosomes other than the four types designated.
[c]Upper limit of relative length for group.
[d]Mean of 24 cells analyzed.
[e]Mean and standard error of 24 cells analyzed.

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6491	CSCRD-103

4. DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 109

10b PRIOR NUMBER CODE: None

11 TITLE: (U) Chromosome function and injury (21)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
012900 Physiology; 014100 Radiobiology; 016200 Stress physiology	11 64	10 67	OTHER | DA

16 PROCURE. METHOD	17 CONTRACT GRANT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
A. GRANT	a DATE 11 64 b NUMBER DA 49-193-MD-65-G 148 c TYPE Y. GRANT d AMOUNT 40,200	PRIOR FY CURRENT FY		

19 GOV'T LAB INSTALLATION ACTIVITY
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20 PERFORMING ORGANIZATION
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TEL 301-927-3800 Ext 210 TYPE A

21 TECHNOLOGY UTILIZATION: Clinical medicine

22 COORDINATION: NA

23 KEYWORDS: Genetics, chromosomes; energy metabolism, adenosinetriphosphate; autoradiography cellular injury; tissue culture; thymidine; stress injury

24 (U) Tech Objective - To complement the in-house work unit on cytogenetic and metabolic determinants in the evolution of cell populations following injury (see Report Code/No. 61130011 3A013001A91C 01 108 dated 30 06 65) specifically, by focusing on selected specific aspects of chromosomal function in such populations.

(U) Approach - The experimental system used is the same as in the in-house work unit. Paralleling the latter's establishment of a baseline for the structural analysis of
25 genome of cell populations in this system, work under this grant centered on the establishment of baselines relative to the functional analysis of the genome. Two functions, chromosome replication and control of energy metabolism, were investigated, the former through H_3 thymidine autoradiography and the latter through luciferin-luciferase determinations of cellular ATP content. Serial determinations as a function of time following media renewal were carried out on normal logarithmically growing cell populations of the L strain mouse fibroblasts.

(U) Progress (Nov 64- June 65) - It was found that chromosome replication follows a cyclical pattern with 15-20 per cent of the cells participating in this process in
26 the first 6 hours, 40% at 12, 30% at 18, and 20% at 24 hours. Omission of media renewal at this point abolishes the cycle and leads to a figure of 0.1% at 42 hours. ATP synthesis was also cyclical with an initial value of approximately 5×10^{-9} micromoles per cell, a maximum of 7 micromoles at 2 hours, decreasing to 6 micromoles at 24 hours and 3 micromoles at 48 hours if media renewal is omitted. These data provide a firm baseline for further work with cell populations surviving stress and injury.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28.	29 OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

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35 EST. FUNDS (In thousands) CFY+1	36

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Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 109, Chromosome function and injury

Investigators.
Principal: Joshua R. C. Brown, Jr., Ph.D.
Associate: Vail, J. M., Glinos, Dr. A. D.

Description:

The proliferation of cells in response to injury has been shown to have considerable significance in replacement of killed or injured cells, in systemic response, as in the immune reaction or the response to a toxic condition, and in the replication of cells which have suffered injury but have not been destroyed. In any such case of rapid replication of cells, either normal or injured, a possibility exists that some "mitotic mistake" may lead to an unequal distribution of chromosomes, or parts of chromosomes to the daughter cells. This possibility is, of course, greatly increased if the cells have been exposed to injury by mutagenic agents such as radiation or certain toxic chemicals. These altered cells, if still capable of proliferation, have been shown to demonstrate an increased variability of chromosome constitution with subsequent generations. Thus there may exist within the site of repair, or perhaps in the system as a whole, a source of cells with an altered genetic complement which may lead to abnormal development, premature ageing, or neoplasia. Furthermore any normal tissue consists of many types of cells, of which one type, through a more rapid rate of proliferation, has attained a position of dominance characteristic of that particular tissue. In the rapid proliferation of cells following injury a possibility exists that some other cell type previously in the minority may be stimulated to multiply at such a rate as to displace the original dominant line, thus altering the character of the tissue. Either of these mechanisms which may alter cellular composition at the site of repair provides a potential for serious consequences since these cells may continue to proliferate even after the normal repair processes have been completed.

It is the objective of this project to complement the in-house work unit on Cytogenetic and Metabolic Determinants in the Evolution of Cell Populations Following Injury, by an investigation of the specific question of variation in chromosome replication and the correlation of such variation with a functional analysis of the genome.

Early analysis of cellular variations in vivo is greatly handicapped by lack of control over the experimental tissue or site and difficulty of analysis on a continuing basis. An in vitro situation, as in cell culture, provides a simpler experimental system for investigation of the phenomena of variation following cell injury, and has been shown to demonstrate the same processes of alteration as those discussed above. Cell cultures also provide an easily analyzed system for study of chromosomal variation and metabolic activity. The cell culture system has, therefore, been selected for this investigation.

Progress:

The experimental system used in this investigation is the same as in the in-house work unit utilizing cell cultures of mouse fibroblast strain L-929 derived from clones derived by the in-house unit. Initial efforts have been devoted to establishment of baselines for comparison before proceeding to study injured cell populations. The problem of replication of the chromosomes and rate of cellular proliferation was investigated through a series of experiments involving pulse labeling of the chromosomal DNA with H^3 thymidine at various times following media renewal. Subsequent analysis by radioautography of the fraction of cells labeled, mitotic rate and fractions of mitotic figures containing labeled chromosomes were made. Investigation of the functional characteristics of the cultures was concentrated on the energy metabolism of the cells through determination of the levels of ATP in the cell by means of the luciferin-luciferase reaction. Investigation of both chromosome replication and ATP level were carried out serially on cultures during the population cycle that suspension cultures of the L strain in the log phase will manifest if medium renewal is omitted.

In a typical experiment along these lines one high and one low density culture with initial cell counts of 986 x 10^3 cells/ml. and 180 x 10^3 cells/ml., respectively, were prepared in Eagle's Minimum Essential Medium plus 10% horse serum, 2 mM glutamine and antibiotics. At 2, 4, 8, 12, 16, 20, 24 and 46 hours following subculture a 10 ml. sample of each culture was transferred to a separate 25 ml. spinner flask on a magnetic stirrer in a 37° C. waterbath. One milliliter of tritiated thymidine solution (2.5 µc/ml., sp. act. 0.36 c/mM) was added and the culture allowed to incubate for ten minutes. The sample was then centrifuged, the cells resuspended in hypotonic media for ten minutes, recentrifuged and fixed, with resuspension, in absolute ethanol-nine parts; glacial acetic acid- one part. Slides were prepared from the fixed cell suspension, dried, stained by the Feulgen method and prepared for radioautography using Kodak NBT-2 bulk emulsion. After the radioautographs were developed, counts were made of labeled cells per 1,000 cells and mitotic cells per 1,000 cells, using phase contrast objectives at 500X magnification. Total cell counts were made on each culture at the same time that samples were removed for radioautography.

The results obtained are given in Table I. As evidenced by incorporation of H^3 thymidine, chromosome replication following media renewal is low (13 - 16.5% of cells are labeled) at two hours after media renewal, rises to a high (40 - 49% of cells are labeled) at 12 hours following media renewal then gradually drops to a lower figure. Samples taken at 46 hours following media renewal show that the high count culture (cell population in excess of 2×10^6 cells/ml.) has ceased to incorporate H^3 thymidine, while at the same time the low count culture (4.2×10^5 cells/ml.) 23.6% of the cells are labeled. This course of labeling indicates that following an initial inhibition of DNA synthesis, because of the manipulation of the cultures during media change, both low and high cell density cultures entered a phase of partially synchronized progressively increasing DNA synthesis and chromosome replication with a maximum between 12 and 16 hours. Beyond this time DNA synthesis and chromosome replication decreased but this decrease occurred considerably faster in the high density culture, obviously because of the exhaustion of components of the medium the concentration of which appear to be limiting for DNA synthesis. Cell division, however, as indicated by the cell counts and the per cent mitotic cells at 20, 24 and 46 hours continued at a high rate and was still present even after DNA synthesis in the high density culture had come virtually to a standstill at 46 hours. The cells dividing at this time had obviously completed their DNA synthesis and chromosome replication earlier.

These observations support the concept that DNA synthesis is only one of the prerequisites for cell division. In order to investigate its exact relationship with another important requirement, the supply of energy, ATP levels representing the intracellular store of energy and medium glucose levels representing the exogenous energy supply were also determined. Initially ATP measurements of L cell suspension cultures by means of an enzymatic method (phosphoglycerate kinase) yielded little data, due to the low cell concentration and to the inadequate sensitivity of the reaction for experimental purposes. These obstacles were overcome by instituting the luciferin-luciferase reaction of Strehler and Totter. This method entails the production of light in direct proportion to the amount of ATP present in a sample. As pilot experiments had indicated that the ATP content of the cells under investigation was extremely small, and therefore light generated by the sample would also be similarly small, an apparatus was designed and constructed which allowed these determinations to be conducted in the presence of room light. This consisted of a 1P21 phototube mounted next to two concentric tubes with sections cut out, so that when the inner tube was rotated, the intact section acted as a shutter. This allowed a test sample to be placed into the inner tube without subjecting the phototube to a great flux of light which might permanently damage the tube.

Table II shows the results obtained with this method in a typical experiment of a design identical with the one previously discussed

except that the initial cell density of 400 x 10^3 cells/ml. was intermediate between the previous ones. The data in Table II show an initial value of 5.5 x 10^{-9} µM ATP/cell immediately after medium renewal and an increase to a value of 7.5 x 10^{-9} µM/cell 2 hours after medium renewal. A relatively high level of ATP is maintained to 24 hours (6.7 x 10^{-9} µM ATP/cell) with a marked drop after 36 hours (4.6 x 10^{-9} µM/cell) and after 48 hours (3.8 x 10^{-9} µM/cell). The cell count at these last two time intervals is comparable to the cell count of the high density culture in Table I. There is a remarkable correlation between the arrest of DNA synthesis at 48 hours shown in Table I and the low ATP, glucose, and growth rate levels at 48 and 60 hours shown in Table II.

The interrelationships suggested by these findings are currently under investigation and form the basis for subsequent work with injured cell populations.

Summary and Conclusions:

To complement the in-house work unit on cytogenetic and metabolic determinants in the evolution of cell populations following injury, specifically, by focusing on selected specific aspects of chromosomal function in such populations:

The experimental system used is the same as in the in-house work unit. Paralleling establishment of a baseline for the structural analysis of the genome of cell populations in this system, work under this grant centered on the establishment of baselines relative to the functional analysis of the genome. Two functions, chromosome replication and control of energy metabolism, were investigated, the former through H^3 thymidine autoradiography and the latter through luciferin-luciferase determinations of cellular ATP content. Serial determinations as a function of time following media renewal were carried out on normal logarithmically growing cell populations of the L strain mouse fibroblasts.

It was found that chromosome replication follows a cyclical pattern with 15-20 per cent of the cells participating in this process in the first 6 hours, 40% at 12, 30% at 18, and 20% at 24 hours. Omission of media renewal at this point abolishes the cycle and leads to a figure of 0.1% at 42 hours. ATP synthesis was also cyclical with an initial value of approximately 5 µM x 10^{-9}/cell, a maximum of 7 µM at 2 hours, decreasing to 6 µM at 24 hours and 3 µM at 48 hours if media renewal is omitted. These data provide a firm baseline for further work with cell populations surviving stress and injury.

TABLE I

Labeling of L-strain cells exposed to 10 minute pulse of H^3 thymidine

Sample No.	Culture	Hours since media change	Cell count (cells/ml.)	Labeled cells %	Mitotic cells %
0	Parent	25	1,653,000	15	0
1	High count	2	986,000	16.5	1.9
1a	Low count	2	180,000	13.0	0.2
2	High count	4	657,000*	15.4	1.7
2a	Low count	4	166,000*	15.1	1.6
3	High count	8	1,040,000	26.3	1.1
3a	Low count	8	239,000	21.0	0.7
4	High count	12	1,160,000	41.0	0.5
4a	Low count	12	340,000	41.4	0.7
5	High count	16	1,270,000	40.1	1.7
5a	Low count	16	278,000	49.1	1.6
6	High count	20	1,385,000	31.4	3.3
6a	Low count	20	303,000	43.1	1.4
7	High count	24	1,616,000	20.5	2.5
7a	Low count	24	356,000	27.0	1.8
8	High count	46	2,139,000	0.1	0.7
8a	Low count	46	419,000	23.6	1.6

* Cell count inaccurate because of cell clumping.

TABLE II

Sample No.	Time in hours	Cell count per ml	Growth Rate*	ATP (10^{-9} µM/cell)	Medium Glucose (µg per ml)
1	0	(medium renewal)		5.50	950
2	2	400,000	-	7.50	900
3	14	617,000	1.54	6.88	803
4	26	857,000	1.39	6.74	644
5	38	1,233,000	1.44	4.59	391
6	50	1,366,000	1.11	3.79	195
7	62	1,320,000	0.97	3.66	33

* Ratio between consecutive cell counts.

RESEARCH AND TECHNOLOGY RESUME

1	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6492	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 110	None

11. TITLE

(U) Fine structure - malaria parasites (09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT COMPL DATE	15. FUNDING AGENCY
010100 Microbiology	09 64	NA	OTHER / DA

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

19. GOV'T LAB INSTALLATION. ACTIVITY	20. PERFORMING ORGANIZATION
NAME Headquarters	NAME Walter Reed Army Institute of Research
ADDRESS U.S. Army Medical Res & Dev Command Washington, D. C. 20315	ADDRESS Washington, D. C. 20012
RESP INDIV Tigertt, Col. W. D. TEL 202-576-3551	INVESTIGATORS PRINCIPAL Sprinz, Col. H. ASSOCIATE Macomber, Lt. Col. P.B. TEL 202-576-2677 TYPE DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
Medicine	NA

23. KEYWORDS

Malaria; plasmodium; microscopy

24. (U) Tech Objective - To define the fine structure of various stages of several malaria parasites during metabolism and as modified by drug action.

25. (U) Approach - High resolution microscopy of Plasmodium fallax, lophura, cathemerium and berghei from tissue culture and in vivo.

26. (U) Progress (Sep 64 - Jun 65) - Striking similarities were demonstrated between the erythro- and the exo-erythrocytic stage of the parasite. A new organelle involved in ingestion was found. Changes in structure resulting from chloroquine action were described.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED K b. NOT RELATED	28.	29. OSD CODE	30. BUDGET CODE
		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A0[illegible]3001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 110, Fine structure malaria parasites

Investigators.

Principal: Col Helmuth Sprinz, MC; Clay G. Huff, Sc.D. (Director, Department of Parasitology, Naval Medical Research Institute, Bethesda, Maryland)

Associate: LtCol Peter B. Macomber, MC; Capt Peter K. Hepler, MSC; Masamichi Aikawa, M.D.; Helen R. Jervis, Sc.D.

Description.

This research is aimed at the morphologic manifestations of the host-parasite relationship in malaria. In the first phase of the investigation the developmental cycle of several species of plasmodia is being investigated with the aid of the electron microscope; in particular, the fine structure of the exoerythrocytic stages of Plasmodium fallax is compared with that of the erythrocytic stages of the same parasite; the feeding mechanism of the erythrocytic stages of P. fallax, lophurae and cathemerium is being studied and is now being compared with that of several species of primate malaria. The investigation of the fine structure of the parasite has been extended to a morphologic study of the antimalarial action of several drugs, principally chloroquine, on P. berghei.

Progress.

In a collaborative study with Dr. Clay G. Huff, our attention has been directed towards elucidating the sequence of development in growing parasites of P. fallax. Erythrocytic stages were obtained from heavily infected turkeys. Exoerythrocytic stages were grown in a tissue culture system derived from embryonic turkey brain cells. Blood or cultures of infected cells were fixed in glutaraldehyde, post-fixed in OsO_4, embedded in Epon and prepared for analysis in the electron microscope.

Merozoites show a highly complex ultrastructure. Their pellicle is composed of three distinct layers; a thin outer membrane, a thicker, interrupted inner membrane, and a layer of microtubules interior to both membrane layers. A conically shaped structure, resembling the "conoid" described by Garnham and co-workers in sporozoites, is located on the anterior tip and appears to be the organelle from which the microtubules radiate. Approximately midway between the anterior and posterior end

of the cell the pellicle shows a circular depression which is smaller but otherwise strikingly similar to the "cytostome" or feeding organelle of the erythrocytic forms. Because of its morphological similarity it is referred to as a cytostome. The cytoplasm contains two large, electron dense, drop-shaped structures, reminiscent of the "paired organelles" of sporozoites described by Garnham and co-workers. These organelles seem to be attached to the conoid. In addition several small densely staining bodies emanate from the region of the conoid. A single crescent shaped mitochondrion is situated at the posterior end of the merozoite usually in close association with a spherical body of unknown significance. The nucleus is centrally located. The matrix of the cytoplasm contains elements of endoplasmic reticulum and ribosomes.

When a merozoite infects a new host cell, it "rounds up," and loses many of its complex structures, including the paired organelles, small dense bodies, unidentified spherical body, conoid, cytostome, and the microtubules and inner thick membrane of the pellicle. The actively growing trophozoite retains only a nucleus, mitochondrion, elements of the endoplasmic reticulum, and ribosomes. The host cell responds to the newly entered merozoite by constructing a thick single membrane completely around it. This membrane is added to as the parasite expands.

The existing cytoplasmic organelles (mitochondria, endoplasmic reticulum, ribosomes and nuclei), increase considerably in number as the uninuclear trophozoite grows into a mature schizont. When segmentation occurs and new merozoites bud off from the central cytoplasmic mass, the structures which were lost by the merozoite upon infection dramatically reappear. The conoid end buds off first, followed in sequence by the paired organelles, small dense bodies, nucleus, and finally the mitochondrion with its associated spherical body. The merozoite is again enclosed in its triple layered pellicle.

Comparisons of the exoerythrocytic stages of P. fallax with the erythrocytic stages show striking similarities in fine structure. The major difference is in the mechanism of feeding. The erythrocytic forms have a cytostome on their surface through which they ingest red cell cytoplasm. Neither cytostomal feeding nor feeding by intracellular phagotrophy has been observed in actively growing exoerythrocytic stages and therefore it is suggested that feeding occurs by some other mechanism probably through diffusion of metabolites from the host cell cytoplasm into the parasite.

Closely related is an electron microscopic investigation of the feeding mechanism of asexual forms and gametocytes of the erythrocytic stages of several species of bird and primate malaria. Instead of

demonstrating intracellular phagotrophy, the commonly assumed feeding mechanism, the erythrocytic stages of all parasites studied so far, showed a specialized pore on the surface of the parasites, being active in the ingestion of red cell cytoplasm. We named this structure "cytostome."

In the process of feeding, the base of the cytostome expands, permitting a large amount of red cell cytoplasm to be taken into the cytostome cavity. Secondary food vacuoles pinch off from the primary food vacuole and appear to be the site of subsequent digestion of the hemoglobin by the parasite. As digestion progresses, the secondary food vacuole becomes denser and aggregations of haemozoin particles appear within the food vacuole. Invagination of the cell pellicle is also produced by the ameboid movements of plasmodia. However, we have no indications that this process leads to a sequestration of host cell cytoplasm. The structure and apparent function of the cytostome in the gametocyte are identical with those in the asexual forms.

We conclude that feeding in erythrocytic forms of species of plasmodium studied by us so far, both in gametocytes and in asexual forms, entails more than simple invagination of the parasite plasma membrane and engulfment of red blood cell cytoplasm by phagotrophy but that it involves a specific organelle, the cytostome, through which the cytoplasm of the host cell is ingested. The investigation of the feeding mechanism of erythrocytic stages of primate malaria is in progress.

The mechanism of antimalarial drug action was studied in P. berghei infected mice. Following i.p. inoculation of chloroquine a characteristic and highly reproducible sequence of morphologic changes occurs in the parasites. By light microscopy clumping of pigment is evident within 30 minutes and reaches a maximum 4 hours after inoculation of the drug. By electron microscopy this process is found to consist of a gathering of individual malaria pigment crystals into large single membrane enclosed vesicles. Portions of parasite cytoplasm, small vesicles containing undigested hemoglobin and unidentified fibrillar material also become incorporated into these same vesicles within the first few hours after chloroquine administration. After 8 hours a progressive diminution of these pigment-containing parasites in the peripheral blood occurs, either as a result of extrusion of the pigment from the parasitized red cells or by removal of these red cells from the peripheral blood and by 48 hours virtually no pigment-containing parasites can be seen.

As early as 5 hours after drug administration occasional parasites are found by electron microscopy to show dissociation of endoplasmic reticulum and ribosomes. This process affects progressively larger numbers of parasites and by 48 hours only rare parasites are found in which this or more advanced forms of cytoplasmic degeneration have not taken place. By 8 hours many parasites show incorporation of abnormally large numbers of food vacuoles in their cytoplasm, a process which also affects progressively larger numbers of parasites as time proceeds. Corresponding to these changes, Giemsa stained blood smears show first inhomogeneity and then progressive loss of cytoplasmic basophilia which is prominent by 24 hours and almost universal and complete by 48 hours while nuclear staining is relatively unaffected. Progressive diminution of average cytoplasmic volume occurs over this same time period. It is also of interest that parasites, in mice whose infection of Plasmodium berghei has relapsed after single dose chloroquine treatment, show a strikingly greater cytoplasmic basophilia than untreated parasites suggesting a rebound phenomenon after release from the repressive effects of chloroquine.

It is hypothesized that chloroquine becomes selectively concentrated in the parasite through chemical binding to hematin, resulting first in focal cytoplasmic damage around the location of hematin in the parasite followed later by more generalized injury as the complexed chloroquine gains access to the rest of the cell and in which ribosomal damage seems to be a characteristic effect.

Summary and Conclusions.

The investigation established several new facts: (1) For the first time the fine structure of both the erythro- and exoerythrocytic stages of the same parasite, Plasmodium fallax, was studied revealing striking similarities except for the feeding mechanism. A heretofore unknown organelle in close proximity to the mitochondrion of the merozoite has been recognized. (2) Studies of the feeding mechanism of the erythrocytic stages revealed the presence of a specialized organelle of the parasite pellicle, the cytostome, through which the cytoplasm of the host cell is ingested. We were unable so far to confirm engulfment of red cell cytoplasm by random invagination of the parasite plasma membrane and phagotrophy. (3) The progression of fine structural changes in parasite cytoplasm of P. berghei following chloroquine administration in vivo was documented and a hypothesis of drug action proposed.

Publications.

None

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA OA6493	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001A91C 01 111	None

11 TITLE (09)

(U) Time sequence, localization and character of the microcirculatory changes in shock

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002600 Biology; 016500 Telemetry	11 64	10 65	OTHER | DA

16 PROCURE. METHOD	17. CONTRACT GRANT a. DATE	18 RESOURCES EST a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
B. CONTRACT	b. NUMBER DA49-193-MD-2683	PRIOR FY	
	c. TYPE A. FPF d. AMOUNT 73,039	CURRENT FY	

19 GOV'T LAB INSTALLATION ACTIVITY	20 PERFORMING ORGANIZATION
NAME Headquarters ADDRESS U.S. Army Medical Res & Dev Command Washington, D. C. 20315 RESP. INDIV Tigertt, Col. W.D. TEL 202-576-3551	NAME Georgetown University Medical Center ADDRESS 3900 Reservoir Road, N.W. Washington, D.C. 20007 INVESTIGATORS PRINCIPAL ASSOCIATE Geschickter, Dr. C.F. TEL 202-333-2000 Ext. 821 TYPE B

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Clinical medicine; telemetry	NA

23. KEYWORDS Anaphylactic shock; blood vessels; shock, traumatic; telemetric thermography

24. (U) Tech Objective - To elucidate the sequence of changes which occur in the microcirculation of major target organs and tissues following the initiation of shock and to follow shifts in blood circulatory volume by telemetric thermography. Results of this investigation will be applicable to the treatment of patients in shock.

25. (U) Approach - Two-hundred-gram male rats injected intraperitoneally with 2 ml of hen egg white develop within 2 to 4 hours peripheral edema involving snout, paws, and scrotum. This anaphylactoid reaction is inhibited by subjecting the animals to a prior tourniquet shock according to the modified Rosenthal method. In a second approach instrumentation for clinical telemetric thermography is being developed.

(U) Progress (Nov 64-Jun 65) - Progress to date has resulted in a novel concept of sequence of circulatory changes in shock.

26. For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1July 1964 - 30 June 1965.

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

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 111, Time sequence, localization and character of the microcirculatory changes in shock as demonstrated by fluorescent and vital dye technics and cinemicrophotography

Investigator.
Principal: Charles F. Geschickter, M.D.

Description.

An investigation of shock which is aimed at a study of morphologic alterations of select target organs in the early phases of reaction to shock producing injuries.

Progress.

The investigation has proceeded in two directions. The possible application of infrared thermography to the study of shock is being explored. Modification of existing radiometric equipment is being attempted to achieve very rapid thermal scanning rates coupled with reasonable resolving power and some form of integrating image display system. Such a device would permit immediate calculation of circulating blood volume since the total heat emission is proportional to the body surface and the blood volume for a given difference between internal and external temperature. The rectal or esophageal temperature, corrected by a factor derived from the peripheral skin temperature, can be used to allow for abnormal peripheral vasoconstriction.

The second line of investigation is based on the working hypothesis that shock is a vascular response to injury which excludes non-vital organs from adequate perfusion and which may prove fatal when ischemia fails and permits toxic products from the denied tissues to re-enter the circulation. In all forms of severe injury, two basic reactions in the microcirculation are opposed: 1) Reactive hyperemia, which increases the perfusion of damaged tissues through vasodilatation and augmented vasoporosity. This is termed phlogistic response and is mediated by histamine and peptides known as kinins, released by injured tissues. 2) Reactive ischemia, which decreases perfusion of damaged tissues and non-vital tissues through arteriolar constriction. This is termed antiphlogistic response and is mediated by adrenalin and other catecholamines.

The possibility that an initial reactive hyperemia is overridden by a reactive ischemia under conditions of severe stress or shock is tested in an experimental model. Rats are subjected to a standardized phlogistic response which is characterized by peripheral edema and which is produced by i.p. injection of sterile egg white. This is the anaphylactoid response of Selye and has been well documented. In another group of rats similarly treated with egg white, hind leg ischemia or tourniquet shock is produced by leaving the tourniquet in place for 4 to 6 hours. No inhibition of egg white edema occurs while the tourniquet is in place but if the tourniquet is released one hour after the injection of egg white and before the edema occurs, shock inhibits this inflammatory response. The morphologic alterations accompanying these responses are being investigated.

Summary and Conclusions.

The study has not sufficiently progressed to reach any conclusions.

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA OA6494	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 112

10b PRIOR NUMBER CODE: None

11 TITLE: (U) Dengue hemorrhagic fever in Thailand (TH)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002300 Biochemistry; 003500 Clinical Medicine	07 64	06 65	OTHER | DA

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

19 GOV'T LAB INSTALLATION ACTIVITY
NAME: Headquarters
ADDRESS: U.S. Army Medical Res & Dev Command
Washington, D. C. 20315
RESP INDIV: Tigertt, Col. W.D.
TEL: 202-576-3551

20 PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012
INVESTIGATOR PRINCIPAL: Cohen, Capt. S.N.
ASSOCIATE: Balankura, Dr. Mayuree
TEL: 202-576-3528 TYPE: DA

21 TECHNOLOGY UTILIZATION: Clinical medicine

22 COORDINATION: NA

23 KEYWORDS: Hemorrhagic fever; shock; virus diseases; acid-base equilibrium; shock and shock intensity; therapy

24 (U) Tech Objective - To accomplish a thorough clinical study of dengue hemorrhagic fever using best available laboratory support in order to outline biochemical abnormalities which would be responsive to intensive clinical management.

25 (U) Approach - The modern methods of measurement of acid-base equilibrium and for early detection of hemoconcentration were utilized to define critical requirements of clinical management of the shock phase of dengue hemorrhagic fever.

26 (U) Progress (Jul 64-Jun 65) - The shock attending the dengue hemorrhagic fever syndrome was shown to be similar to other forms of shock in that hyponatremia, metabolic acidosis, cellular damage, and tissue hypoxia were observed. These abnormalities were correctable by the established fluid and electrolyte regimen used for other forms of shock. Hyponatremia appears to play a role both in the development of shock and in the patient's response to therapy. Plasma protein therapy was frequently necessary in treatment of the shock phase. Therapy should be limited to supportive measures since there is no specific treatment and the potent drugs may be dangerous.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a COMSEC OR COMSEC RELATED X b NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) FY ...	36

DD FORM 1498 AUG 64 — Items 1 to 36 identical to NASA Form 1122. REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 112, Dengue hemorrhagic fever in Thailand

Investigators.
Principal: Capt. Sanford N. Cohen, M. C.
Associate: Mayuree Balankura, M. D.
Chaiyan Kampanart-Sanyakorn, M. D.

Description:

The object of this study was to accomplish a thorough clinical evaluation of dengue hemorrhagic fever, using the best available laboratory support in order to outline biochemical abnormalities which would be responsive to intensive clinical management.

Progress:

Following the previously reported survey of possible nutritional problems which could possibly be associated with reported differences between mortality of Thai and European cases of hemorrhagic fever, the services of Dr. Sanford Cohen were requested by the Thai Government to establish a facility for detailed clinical study of the disease.

Approach - The first major epidemic of dengue hemorrhagic fever to occur in Thailand began in July 1958. Since that time, there have been more than 10,000 cases hospitalized in Bangkok during the annual epidemics and approximately 1,000 have died. The Thai Hemorrhagic Fever Study Center was organized in 1964, under the

coordination of the Minister of Public Health in order to complete the description of dengue hemorrhagic fever as it occurs in Thailand and test a therapeutic regimen for serious cases. This report will summarize the findings in 149 patients studied at this special clinical research unit between July and September, 1964, and discuss the regimen used at the Center.

There were 70 male and 79 female patients admitted to the Center during its operation. These children ranged in age from five months through 17 years (median age, 5 years). The diagnosis of "Thai" hemorrhagic fever was based on the finding of shock at the time of admission or upon a combination of such findings as fever, a history of vomiting and anorexia, a petechial rash, a positive tourniquet test and diffuse lymphadenopathy. There was no sign of bacterial infection to explain any of these findings.

A diagnosis of shock was made either when the blood pressure was not detectable or when the pulse pressure was less than 20 mm Hg in a patient who had cold, clammy skin, tachycardia, restlessness and stupor. This diagnosis was always confirmed by at least two members of the medical staff.

Patients were observed both in the hospital and again 7-10 days after discharge. The period of observation usually spanned 15-20 days. Studies performed as part of the routine admission evaluation are listed in Table 1. The hematological, serological and chemical studies, along with the urinalysis, were routinely repeated throughout the period of observation. Standard clinical chemical and serological methods were adjusted to microvolumes so that determinations could be performed on minimal quantities of blood.

A four-fold rise in dengue hemagglutination-inhibition titer during the period of observation or a titer of 1:640 or greater was considered proof of dengue infection. All patients not meeting these criteria were assumed to have had a non-dengue illness and were grouped together as a "control" for the purpose of comparison with the dengue cases. Virus isolation and identification studies are not complete and will be published separately.

Since bleeding severe enough to lower the hematocrit occurred in only one case, the level at which the hematocrit stabilized was assumed to approximate the level prior to the illness. The ratio

$$\frac{\text{Highest Hct - Recovery Hct}}{\text{Recovery Hct}}$$

was computed for the surviving cases. A ratio of 0.2 or greater was taken as evidence of significant hemoconcentration.

Results- There was serological evidence of dengue infection in 123 of our cases. Chikungunya infection was serologically diagnosed in five of the non-dengue controls while the remainder had a non-specific (viral) illness. 19 of the dengue patients were admitted in shock and four others developed shock in the hospital. None of the controls developed shock. Three patients died during the course of the study (2.0%).

The clinical features in our cases at the time of admission are summarized in Table 2. The differences between the dengue group and the non-dengue controls are not statistically significant.

The incidence of abnormal laboratory findings in the various groups is shown in Table 3. The differences between the incidence in the shock group and in the other groups are all highly significant with the exception of proteinuria where no significant difference was observed. The difference between the incidence of hyponatremia, acidosis, mild azotemia, elevated transaminase levels and hemoconcentration in the non-shock dengue group and in the controls is not significant. However, the difference between the frequency of hypoproteinemia in these two groups is significant.

Serum bilirubin was transiently elevated in one case. Blood glucose concentration was normal at the time of admission. Transient, heavy proteinuria (2+ or greater) was seen in 11 patients, but was as common among the non-shock groups as among the shock patients. Admission electrocardiograms showed no abnormalities which could be related to the patients' acute illness. Group A, beta hemolytic streptococci were recovered from routine throat cultures in four cases and from a skin infection in a fifth. There was no bacteriological evidence of a systemic infection at the time of admission or of a serious bacterial complication in any case.

The admission and terminal findings in the three fatal cases are listed in Tables 4 and 5. In all three cases, death appeared to be related to the cardiac effects of hyperkalemia. Post-mortem examinations were carried out in all three cases. They all had serous effusions, widespread edema and congestion of tissues at autopsy. They each had necrosis of individual liver cells with the formation of Councilman-like bodies. There was intense reticuloendothelial activity in the spleen, lymph nodes and thymus of each child. There was pronounced oozing of serous fluid from the tissues during the autopsies. One child had a large, fresh subarachnoid hemorrhage at the base of the brain. There were subendocardial petechiae and

hemorrhagic areas within lymph nodes in all three. There was narrowing of the zona glomerulosa, but the adrenals were otherwise normal.

Treatment - Patients were given symptomatic, supportive therapy, since there was no specific treatment available. Patients in shock and all others who appeared clinically dehydrated were given a rapid infusion of Ringer's lactate solution followed after one hour by a solution of one part Darrow's potassium lactate diluted with either two or three parts 5% glucose in water. All patients were assumed to be 5% dehydrated for the purpose of calculating replacement therapy. These initial solutions were followed by routine maintenance therapy until the patients were well enough to resume a normal oral intake. Oxygen was administered to all patients in shock.

A change in hematocrit over a period of a few hours, in the absence of blood loss or blood transfusion, can be assumed to reflect a change in the plasma volume. Since hemoconcentration was a frequent abnormality in the shock group, the progress of therapy was followed by means of frequent microhematocrit determinations. All of the abnormalities associated with shock, including the hemoconcentration, were corrected without complication in five cases. In the other 18 patients, however, microhematocrit values ceased to fall or actually rose during fluid replacement therapy. When this failure to respond to therapy was noted, a plasma protein solution*was added to the regimen. The dose of Plasmanate was 10-20 cc/kg/hr. In no case was a total dose in excess of 60 cc/kg required. The addition of this solution resulted in a prompt decline in hematocrit in all surviving cases. Death occurred too soon after the institution of such therapy in the fatal cases for its effect to be evaluated.

The overbreathing, so characteristic of metabolic acidosis, was rapidly corrected in most cases by following the usual regimen. In two cases, however, the children continued to show signs of severe acidosis and sodium bicarbonate was added to the regimen. When indicated by the patient's condition, 1-2 cc/kg of a 3.75% sodium bicarbonate solution**was slowly injected intravenously every 15 minutes until clinical improvement was noted.

Agitated children were controlled with physical restraints or with chloral hydrate or paraldehyde. Fever was controlled by hydrotherapy (sponging) or by a combination of hydrotherapy and oral acetaminophen***.

* Plasmanate:5% plasma protein solution. Cutter Lab Inc. Berkeley, Calif.
** Abbott Lab, North Chicago, Ill.
*** Tylenol: McNeil Lab, Inc. Fort Washington, Pa.

Penicillin was administered to the children with streptococcal infection. Barbiturates, phenothiazines, salicylates, vasoactive drugs, broad spectrum antibiotics and adrenocortical steroids were not given to any patient.

Discussion - Epidemics of dengue fever have been characterized by a high rate and morbidity. This classical form of dengue-induced illness, even in its most severe form, has had a fatality rate of less than 1%. Dengue hemorrhagic fever, on the other hand, has been associated with an average case fatality rate of approximately 10% in Thailand and considerably higher in other countries of Southeast Asia.

Death from hemorrhagic fever in Thailand has been limited almost exclusively to children who have dengue infection and who develop shock during the course of the illness. The pathophysiology of the syndrome, especially the shock which leads to death in many cases, has been only poorly understood and therapy has therefore been difficult to plan. Since effective dengue immunization, total Aedes aegypti eradication and specific anti-dengue virus therapy will not be available within the foreseeable future, our emphasis was upon understanding the pathophysiology of the syndrome so that a therapeutic regimen could be planned which might minimize fatalities during future epidemics.

Hypoproteinemia occurred in the non-shock dengue group with statistically greater frequency than in the controls. Thus, hypoproteinemia seemed to be a part of the syndrome of dengue infection itself. The remaining abnormalities occurred with similar frequency among the control patients and the non-shock dengue group. This indicates that hyponatremia, acidosis, azotemia, transaminase elevations and hemoconcentration might all represent non-specific metabolic changes due to infection rather than changes due to any specific viral illness. Since shock itself is frequently associated with these same physiological abnormalities, it was not surprising to find that the shock group had the highest incidence of these findings.

The basis for the seemingly paradoxical finding of a decreased total serum protein concentration in association with an increased hematocrit in dengue hemorrhagic fever is not fully explained, but, along with dehydration, it appears to play a major role in the shock seen during the illness. The dehydration produced by the dengue-induced anorexia and vomiting does not appear severe enough to lead to shock in any case. We therefore postulate the following sequence of events in the production of shock in dengue hemorrhagic fever.

There is a net lowering of the protein concentration of the plasma when protein-rich plasma water leaves the vascular space due to capillary damage and protein-poor extracellular water enters this space to maintain the circulating blood volume. The resultant lowered oncotic pressure reduces the movement of extracellular water into the vascular space and thereby limits the ability to compensate for hypovolemia. If dehydration has lowered the volume of extracellular water available for this (incomplete) maintenance of the circulating volume, shock occurs. Acidosis and hypoxia occur during shock and probably contribute to the peripheral vascular collapse. When these abnormalities reach critical levels, they contribute to a rapid rise in extracellular potassium concentration. This hyperkalemia disrupts normal cardiac functioning and death occurs. Hypoproteinemia adversely affects therapy by allowing the administered fluids to leave the vascular space so rapidly that shock is not relieved. Under these circumstances, tissue edema is exaggerated and hemoconcentration persists.

Intravenous therapy, designed to relieve the dehydration, shock and acidosis, should probably be the initial therapy in all cases of dengue hemorrhagic fever. If there is evidence of persistent hemoconcentration during fluid therapy, a plasma protein solution should be administered in order to relieve hypoproteinemia. Emergency treatment with single doses of calcium gluconate, glucose and insulin and sodium bicarbonate failed to keep our patients alive after hyperkalemia appeared. A constant infusion of calcium gluconate might relieve the cardiac effects of the hyperkalemia long enough for more definitive therapy (e.g. ion exchange resins) to be effective and it should probably be tried.

Whole blood is probably contraindicated in hemoconcentrated states and should be reserved for those patients in whom there is laboratory evidence of red blood cell loss. Adrenocortical steroids, broad spectrum antibiotics, hypnotics, tranquilizers and vasoactive drugs should probably be avoided, since they are all potentially dangerous agents.

From this study the following observations are extended:

1. The dengue hemorrhagic fever shock syndrome is similar to other forms of shock in that there is evidence of hyponatremia, metabolic acidosis, cellular damage and tissue hypoxia. These abnormalities can be corrected by the same type of fluid and electrolyte replacement regimen used for other forms of shock.

2. Hypoproteinemia appears to play a role both in the development of the shock and in the patients' response to therapy. Plasma protein therapy is frequently necessary in the treatment of the shock phase of hemorrhagic fever.

3. Therapy should probably be limited to supportive measures, since there is no specific treatment and potent drugs may be dangerous. Supportive therapy may offer a means to reduce the mortality from dengue hemorrhagic fever.

Table 1

Studies done on 149 hemorrhagic fever patients at the time of admission*. Bangkok, 1964

Sodium	Potassium	Chloride
CO_2	Urea nitrogen	SGOT
SGPT	Glucose**	Bilirubin**
Total serum protein	Serum electrophoresis	Hematocrit
Hemoglobin	White blood count	Differential count
Throat culture	Nasopharyngeal culture	Blood culture
Urine culture	Stool culture	Urinalysis
Chest X-ray	Electrocardiogram	Virus isolation attempt
Arbo-virus serology		
Stool exam for parasitic infestation		

* Most studies were repeated periodically.
** Not done in all cases.

Table 2

The frequency of various clinical findings in 123 dengue and 26 non-dengue cases at the time of admission. Bangkok, 1964

Finding	Dengue	Non-dengue
Hepatomegaly*	6.5%	3.8%
Rash(all)	56.9%	53.8%
Petechial rash	43.1%	26.9%
Palpable lymph nodes	72.4%	65.4%
Injected tonsils & pharynx	97.6%	100.0%
Anorexia	90.2%	69.2%
Vomiting	66.7%	42.3%
Shock	18.7%**	0.0%

* Liver edge at least 2 cm below right costal margin.
** Includes 4 patients who developed shock after admission.

Table 3

The frequency of various abnormal laboratory findings in 149 cases of hemorrhagic fever. Bangkok, 1964

Finding	Dengue(123) Shock(23)	Non-shock(100)	Control(26)
Sodium 135 mEq/L*	65.5%	16.0%	15.4%
CO2 15 mM/L* (with acid urine)	62.0%	18.9%	11.5%
Serum urea nitrogen* 20 mg/100 cc	62.0%	22.0%	11.5%
SGOT 150 S.F. Units	56.5%	16.0%	3.9%
SGPT 100 S.F. Units	30.5%	8.1%	0.0%
Proteinuria 2+	8.6%	8.0%	3.8%
Hemoconcentration**	95.5%	31.7%	11.8%
Total serum protein 5.5 g/100 cc	78.5%	31.0%	7.7%

* At the time of admission.

** $\frac{\text{Highest Hct-Recovery Hct}}{\text{Recovery Hct}}$ 0.2

Table 4

Laboratory findings in three fatal cases of dengue hemorrhagic fever at the time of admission. Bangkok, 1964.

Finding	Case No. 41	Case No. 53	Case No. 80
Hematocrit (%)	44.0	39.0	43.0
Sodium 130-135 mEq/L	+	+	+
CO_2 15 mM/L	+	+	+
Total serum protein 5.5 g/100 cc	+	-	-
SGOT 150 S.F. Units	+	+	+
SGPT 100 S.F. Units	-	-	+
Serum urea Nitrogen 20 mg/100 cc	+	+	+
Potassium 6.0 mEq/L	-	-	-
Electrocardiographic evidence of hyperkalemia	-	-	-

Table 5

Laboratory Findings in Three Fatal Cases of Dengue Hemorrhagic Fever Just Prior to Death, Bangkok, 1964

Finding	Case No. 41	Case No. 53	Case No. 80
Hematocrit (%)	47.0	44.0	41.0
Sodium 130-135 mEq/L	+	+	-
CO_2 < 15 m M/L	+	-	-
Total serum protein 5.5 g/100 cc	+	+	+
SGOT > 150 S.F. Units	+	+	+
SGPT > 100 S.F. Units	+	+	+
Serum urea nitrogen > 20 mg/100 cc	+	+	+
Potassium > 9.5 mEq/L	+	+	+
Electrocardiographic evidence of hyperkalemia	+	+	+

Summary and Conclusions:

A field study of the clinical management of Dengue Hemorrhagic Fever (DHF) has been accomplished in Thailand. It was established that the shock phase of DHF is similar to other forms of shock in that hyponatremia, metabolic acidosis, cellular damage, and tissue hypoxia occur. These abnormalities can be corrected by the same type of fluid and electrolyte replacement regimen used for other forms of shock.

Publications:

Balankura, M., Valyasevi, A., Kampanart-Sanyakorn, C., and Cohen, S., Dengue Infection in Thai Children. A Preliminary Report from Thai Hemorrhagic Fever Center, Bangkok. Presented at the WHO Interregional Seminar on Mosquito-Borne Hemorrhagic Fevers, Bangkok, Thailand. 19-25 October 1964.

Balankura, M., Valyasevi, A., Kampanart-Sanyakorn, C., and Cohen S., The Treatment of Dengue Shock Syndrome. A Preliminary Report from Thai Hemorrhagic Fever Study Center, Bangkok. Presented at WHO Interregional Seminar on Mosquito-Borne Hemorrhagic Fevers, Bangkok, Thailand. 19-26 October 1964.

Cohen, S. N., Dengue Hemorrhagic Fever in Thailand. Presented at the Tri-Service Pediatric Seminar, Walter Reed Army Medical Center, 3-5 March 1965.

RESEARCH AND TECHNOLOGY RESUME

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19 GOV'T LAB INSTALLATION ACTIVITY
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21 TECHNOLOGY TILIZATION	22 COORDINATION
Public health	NA

23 KEYWORDS: Respiratory infection; stress; endocrine response; hormones; viruses; sociology; personality type.

24 (U) Tech Objective - Definition and evaluation of various environmental and personal factors which contribute to physical and psychological stress experienced by military personnel, and determination of how these affect the overt clinical manifestations of naturally acquired infections. When factors are defined, efforts to modify clinical manifestations by modification of environment or human response to it are made.

25 (U) Approach - Endemic overt diseases in military populations are identified and studied for microb. etiology, variation in clinical manifestations. Environment in which they occur is defined. These findings are correlated with patients' immunological susceptibility, physiological responses to environment and its stresses, and with personality types, and social backgrounds. Factors suspected of influencing disease severity are evaluated in controlled experiments.

26 (U) Progress (Oct 64 - Jun 65) - Acute adenovirus respiratory disease (ARD) was found to be associated with significant changes in thyroid and adrenal function in many of 48 Army recruits studied linearly through 8 weeks of basic training. Disability (hospitalization) for ARD appears to be related to previous cultural experience of the patient in "use" of disease to avoid stressful situations.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a COMSEC OR COMSEC RELATED / NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
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Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 113, Effects of Physiological and Psychological Stress upon Infection and Disease

Investigators.

Principal: Capt Peter G. Bourne, MC; John W. Mason, M.D.; Maj William E. Datel, MSC; Lt Col Edward L. Buescher, MC;

Associate: Maj Bernard J. Wiest, MSC; Charles F. Gieseking, M.A.; Capt Anton O. Kris, MC; Myron L. Belfer, M.D.; John A. Jones, Ph.D.; Malcolm S. Artenstein, M.D.; Capt John A. Stephenson, MC; Capt Robert E. Blount, MC.

Description.

To define and evaluate various environmental and personal factors which contribute to physical and psychological stress experienced by military personnel, and to determine how they affect the overt manifestations of naturally acquired infections.

Progress.

1. Effect of Physical and Emotional Stress on Acute Respiratory Disease in Army Recruits. Two striking features of basic training of Army recruits are the severe physical and emotional stresses to be endured by the recruits, and the high incidence of acute respiratory disease. During the winter of 1965 an interdisciplinary approach to determine whether these two factors are related was undertaken. The training environment and the subjective response of the men to it was defined by the staff of the Departments of Psychology and Psychiatry. The endocrine response of the subjects was studied by the Department of Neuroendocrinology. Disease and infecting agents were studied by the Department of Virus Diseases.

At this time, endocrinologic studies are only partially complete and the psychologic-psychiatric data is incompletely analyzed. For the purpose of this report, only preliminary illness, virologic and bacteriologic data are presented.

a. Techniques, experimental design. Forty-eight new recruits comprising a single platoon at Fort Dix, New Jersey, were studied continuously from their arrival at the reception station in January 1965 until completion of their basic training in March 1965. Serial observations,

testing, interviews, and specimen collections were carried out by the psychiatrists, psychologists, social workers and neuroendocrinologists to define and measure emotional and physical stress. The virologists carried out the following procedures to define the etiology and rank the severity of the illnesses encountered.

Body temperature. Oral temperatures were taken daily on each man throughout the first five weeks of basic training and on weekdays thereafter. This was done with telethermometers and accomplished during the early evening.

Throat washings, using Hank's BSS with 0.4% bovine albumin, were taken on each man on arrival at the reception station and twice weekly thereafter during basic training. These were stored at -70° and returned to WRAIR for routine virus isolation procedures.

Throat swabs for bacterial pathogens were obtained once weekly, inoculated on the appropriate media and delivered to the Department of Bacteriology, WRAIR, for identification and typing of meningococci and beta hemolytic streptococci.

Serum specimens were collected weekly from each man for serologic studies.

ENT examinations were done on each man twice weekly to document the presence or absence and to determine the severity of clinical illness. Those men found febrile or more than minimally symptomatic were examined completely at that time by one of the investigators and appropriate additional specimens collected. Those subjects who met sick call or required hospitalization were followed closely by the investigators but in all instances treatment and disposition of the patient was at the exclusive discretion of the regular Walson Army Hospital physicians, including the decision as to whether or not hospitalization was indicated.

b. Virus isolation. Table 1 indicates the number of individuals by week of training from whom virus isolates were made. Polio isolates were presumed related to preceding oral vaccine administration. Of the adenovirus 4 isolates in weeks 3 and 4, 3 men in each group also had adenovirus 4 isolates the previous week. No individual yielded positive adenovirus 4 isolates for longer than a seven-day span.

c. Serology. Table 2 lists by week the number of men demonstrated to have circulating antibodies to Adenovirus 4. Results were obtained by type specific neutralization tests except in a few instances where isolation of the specific virus during a clinical illness followed by four-fold rise in CF antibodies was accepted.

Table 1 Number of Subjects with Virus Isolations

	Training Week								
Virus	0	1	2	3	4	5	6	7	8
Adv.#4			5(1)*	23(2)*	12(1)*	1(2)*			
Adv.#3					1				
Paraflu I		1(1)*							
Herpes S.	1	1			1				
Polio #I		3	1						
Rubella						(1)*			
Total Strength	48	48	48	48	46	41	40	39	39

*Figures in parentheses indicate additional tentative viruses recovered

Table 2 Development of Serologic Immunity by Week

	Training Week									
Number of Individuals with Onset of Antibody Development	0	1	2	3	4	5	6	7	8	Total
	5	0	0	11	13(2)*	13(1)*	2			47

*Figures in parentheses reflect additional tentative serological conversions

d. Bacteriology. Table 3 demonstrates the number of men by week with throat cultures positive for meningococci and/or beta hemolytic streptococci. During the training cycle no systemic meningococcal disease was noted and only one man had an illness that seemed clearly to represent streptococcal pharyngitis. This illness occurred during the

seventh week of training, resulted in hospitalization and rapid response to penicillin therapy. Type G beta strep was isolated from his pharynx at the onset of illness whereas all of his previous beta strep cultures had been negative.

Table 3 Bacterial Isolates During Basic Training by Week

Number of Positive Cultures	Week of Training 0	1	2	3	4	5	6	7	8
Meningococcus									
Sensitive*	20	14	16	20	30	37	24	29	21
Resistant	0	0	2	3	1	1	10	7	8
Total	20	14	18	23	31	38	34	36	29
Beta Strep									
Group A	2	2	1	1	1	1	1	1	1
Group B	1	2	3	5	2	4	1	2	
Group C	2	1	1	1	1	1	1	1	1
Group F		2	1					2	2
Group G	1		1	1	1		1	1	
Group AG	1								
Group BG		1		1			1		
Group BF									1
Group GF									1
Not A-H							2	3	8
Total	7	8	7	9	5	6	7	10	14
Total Number of Subjects	48	48	48	48	46	42	40	42	41

*To 0.1 mgm% sulfadiazine in the medium.

e. Clinical observations. Table 4 reflects by week the distribution of clinical illness as observed. The following criteria for classification were used:

Asymptomatic.

AF. Afebrile but symptomatic or with objective findings of upper respiratory infection.

MF. (Mild febrile URI). Fever of 37.5 - 38.4°C for at least two days with objective findings of upper respiratory infection for at least two days.

SF. (Severe febrile URI). Fever of greater than 38.5°C on one day and greater than 38.0°C on a consecutive day with physical findings of upper respiratory infection on those two days.

Table 4 Incidence and Clinical Severity of URI by Week

No. of Men in Each Illness Category	Training Week								
	0	1	2	3	4	5	6	7	8
Asympt.	37	26	25	21	43	38	40	38	38
AF	10	16	13(5)	11(9)	1(1)				
MF		6	3(3)	3(3)	2(2)	1		1*	1
SF	1**	1(1)	7(7)	13(11)		2***			
Admitted to Hospital		3	7	11		2***		1*	
Total Platoon Strength	48	48	48	48	46	41	40	39	39

( ) Proven adenovirus infections
* Streptococcal Pharyngitis
** Parainfluenza I (tentative)
*** One rubella and one pneumococcal pneumonia

f. Current interpretations of infection patterns. Of the original 48 men in the study group, 9 were lost from study for a number of reasons. These 9 recruits amounted to 19% of the original group. Most of these were recycled because of illness; an occasional individual was either recycled of discharged from service for other more personal reasons. Five (10%) were seen to be immune on the basis of neutralizing antibody in the original serum specimen taken. The attack rate in the remaining 43 susceptibles by adenovirus infection was 100%; this had occurred by the 6th week of the cycle. The epidemic curve for adenovirus infection began

in the first week of the training cycle, increased during the second, and reached a peak during the third week, a few cases were noted during the fourth week and then the epidemic was essentially over. Table 4, relating the incidence and clinical severity of respiratory illness to week of training, shows that afebrile respiratory illnesses were quite common during the pre-training period and during the first three weeks of the training cycle. After this time, essentially no afebrile respiratory disease occurred in the group. Febrile illnesses, classified as "mild", occurred with about the same frequency in the first, second, third and fourth weeks of training, whereas severe febrile respiratory illness occurred sporadically except during the second and third weeks when large numbers of cases were seen. The incidence of adenovirus infection correlated very well with febrile respiratory illness and exceptionally well with the severe respiratory illness group in which, during the second and third week, 18 of the 20 cases were found to have a simultaneous adenovirus infection. Thus this data suggest that in this group of men adenovirus infection almost always resulted in a febrile respiratory illness. The lack of association of afebrile respiratory symptoms with adenovirus infection is of interest. Only a few of the cases in the early weeks of training were found to have infection by a parainfluenza virus and no influenza occurred. Further studies of the throat wash material from this group of individuals is under study in a variety of tissue culture lines in the hope that other agents, such as the rhinoviruses and the parainfluenza viruses, might be uncovered.

The striking lack of respiratory disease from the 4th to the 8th weeks of the training cycle deserves some comment. It is conceivable that bias on the part of the observers in recording symptoms may have played a role, although this would appear unlikely since even during that period of time the same protocol was followed. It is possible that this cessation of respiratory symptomatology and illness is related to psychologic or other type of adaptation to the environment and further analysis of the psychologic, psychiatric and endocrinologic data may be of assistance in evaluating this hypothesis.

Forty-two per cent of the study group were pharyngeal carriers of meningococci upon arrival at Fort Dix. None of the 20 strains were found to be sulfadiazine resistant. The percentage of positive individuals fell off slightly during the first and second weeks, then began to increase. In the 7th week 86% of the individuals tested were now carriers, and in the 8th week, 38% of the strains isolated from the nasopharynx were of the sulfa-resistant variety. This was in the absence of any prophylactic administration of drug. It is not possible to state whether

any of the observed respiratory illness was related to the meningococcal infection isolations; however, it can be said that no systemic meningococcal infections occurred in this group, and that rising carrier rates tended to parallel the increase in overt respiratory disease. Further studies of the immune response to the meningococcus are currently underway in the laboratory.

Hospitalization statistics for the group indicate that there were 24 hospital admissions for respiratory infection during the study, representing 21 individuals, three of whom were hospitalized on more than one occasion. Thus, 44% of the platoon were hospitalized at some period during the training cycle. All of the hospitalizations were in the febrile respiratory infections. Therefore, although hospitalization rates do not reflect the total amount of respiratory disease in recruits, it may very well be a good index of severe respiratory infection, which in turn, in this particular study, was primarily associated with adenovirus infection.

g. Hormonal Balance Preceding Medical Illnesses. Considerable indirect evidence suggests the possibility that stress-related changes in overall hormonal balance may influence the course or have a role in the pathogenesis of certain medical illnesses. As an initial step in the evaluation of this untested hypothesis, daily 24-hour urine samples and weekly blood samples were collected in a platoon of soldiers during the basic training period at Fort Dix this past winter. Seventeen of these subjects developed relatively severe respiratory infections during the first three weeks of the cycle. On 14 of these subjects, urine samples for hormone analysis are available for from 7 to 14 days preceding the onset of fever and symptoms. Preliminary results indicate that substantial hormonal changes occur in most subjects well in advance of the onset of illness and that one or more characteristic patterns of change can be identified. The most common pattern of change seen prior to onset of illness is characterized by a declining thyroid hormone level, a rising 17-OH-CS and epinephrine level, a sustained norepinephrine elevation, and a declining estrogen level. Urinary testosterone, androsterone, etiocholanolone and dehydroepiandrosterone levels and plasma insulin levels have yet to be analyzed. A variety of control measurements are also being made, including those on the 12 subjects with the lowest incidence of illness during the basic training period and those on the hospitalized subjects following complete recovery in the sixth and seventh weeks. Documentation of the illness symptoms and signs along with virological and immunological data collected by the Department of Virus Diseases is available for correlation

as is a considerable amount of psychiatric, social, and clinical psychological data on these subjects. This project promises to yield a great deal of information which should make it possible to evaluate the feasibility of and the urgency for further studies involving this new experimental approach.

In connection with a study of hormone patterns prior to respiratory illness in basic trainees at Fort Dix in February 1965, plasma and urinary hormone samples were obtained on human subjects before, during, and following a variety of psychologically stressful events. Preliminary findings suggest that humans frequently show a stereotyped hormone response pattern in association with emotional distress which is very similar to that which is now well documented in our studies with the monkey. Considerable psychiatric and psychological data is also available on these subjects so that any deviations from the stereotyped response pattern may be viewed in terms of personality factors or previous experience differences between subjects.

Finally, continuous 24-hour urine samples and frequent blood samples have been obtained before, during, and following the febrile period in 17 Fort Dix recruits hospitalized for respiratory adenovirus infections this past winter. The correlation of changes in the overall pattern of about 12 hormones with the intensity and duration of fever, pharyngitis, cough, and rhinorrhea is being determined. Of particular interest are three subjects who progressed to viral pneumonia during hospitalization.

h. Sociologic Background and Personality Types. The sociologic backgrounds of 48 men in the same platoon at Ft. Dix were studied and the project lasted for the duration of their cycle through basic training. Collection of data from interviews, questionnaires, sociometric tests and behavioral observations has been completed. The large quantity of data which evolved from this effort is currently being analyzed.

Initial evidence suggests that social determinants play the major role in influencing the use of the sick role in basic training, and that certain administrative features built into the system such as recycling can exert an overwhelming influence in deciding whether a recruit seeks medical help or not.

Considerable progress has also been made in elucidating the overall process of basic training, and in better defining the interaction of different personality types under stress in determining behavior patterns.

i. Affective Arousal in Basic Training. The purpose of this study is to chart the changes in affect undergone by a platoon of recruits from inception to completion of the Army basic combat training (BCT) process. In addition, the study traces the shifting levels in dysphoric affect during an early 10-day period of the BCT cycle on the same platoon of recruits.

The data of this study were gathered in connection with a broad interdisciplinary exploration undertaken by representatives of several Departments of WRAIR at the United States Army Infantry Training Center, Fort Dix in the early months of 1965. Although in its most general sense this combined investigatory effort concerned itself with a quest for physiological and psychological correlates of respiratory disease in recruits, one of the important parameters which required identification was the characteristic pattern of subjectively experienced "stress" during the BCT cycle. Repeated, spaced queries, via a self-reporting device, into the moods of the trainees as they moved from civilians to greenhorns to seasoned recruits provided the approach toward specification of the high and low points of experiential distress. Inasmuch as Marlowe of this Division has in the past already characterized the peak period of stress during basic training by the use of participant-observation methodology, the study also afforded an opportunity for construct validation of the particular psychometric instrument utilized.

The Multiple Affect Adjective Check List (MAACL), a list of 132 adjectives with instructions to the subject to check those words which describe how he has felt during a specified time period, was used in a repeated measures design. Weekly time-set forms were administered nine times to a platoon of men. Daily time-set forms of the MAACL were administered consecutively for 10 days to the same subjects during the first part of the cycle. Analyses of variance performed on each set of measures showed highly significant occasions effects. Standard scores were devised for the three sub-scales of Anxiety, Depression and Hostility and permitted comparisons between affects at different stages of the cycle and upon different daily activities.

The results provide a graphic representation of the pattern of distress for the BCT cycle as announced by the platoon of soldiers studied. The peak of psychological discomfort is near the midpoint of the cycle, before the dawn of the fourth week. Results from the 10 daily time-set administrations show remarkable sensitivity of the MAACL to events such as weekend days and days spent on KP. On several points the study increases the construct validity of the MAACL. A manuscript is nearing the final stages of preparation.

Summary and Conclusions.

An interdisciplinary evaluation of roles played by psychological and physical stress upon the occurrence of infection with respiratory viruses and disease caused by them was commenced with 48 recruits in a single platoon undergoing basic training at Fort Dix, New Jersey February - March 1965. This study as yet incomplete has shown; a) that adenovirus induced respiratory disease occurred as predicted in the 2nd and 3rd week of basic training; 27 of 48 men were so affected: 18 were hospitalized. Adenovirus infection rates were 100%; 43 of 43 susceptibles presenting evidence for infection; b) hormonal pattern changes were detected in certain men upon arrival at Fort Dix; these patterns permitted arbitrary classification of subjects, and are being correlated with patterns of infection and disease. Similarly changes in hormones were observed to precede severe illness in several subjects by 3-6 days. c) sociologic and personality types were determined for each man; these will be correlated with neuroendocrine and infection patterns: d) affect of recruits was found to be variable but influenced by events occurring in basic training. Peak psychological discomfort was observed late in the 3rd week of training.

Publications - None

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6496	CSGRD-103

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30 06 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 114

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11. TITLE: (U) Malarial antigens (09)

12 SCIENTIFIC OR TECH. AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
010100 Microbiol., 002600 Biol.	07 64	NA	OTHER DA

16 PROCURE. METHOD	17 CONTRACT GRANT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
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19 GOV'T LAB INSTALLATION ACTIVITY
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TEL: 202-576-3544 TYPE: DA

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Serology	NA

23 KEYWORDS: Malaria; plasmodium; antibody; antigens; complement; complement fixation tests; parasitic diseases; serodiagnosis; serology; biochemistry

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.

25. (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.

26. (U) Progress (Jul 64 - Jun 65) - Lysis of heavily infected blood with CO_2-saturated distilled water followed by differential filtration yielded parasite material essentially free of blood components. Promising CF antigens have been obtained by passing crude plasmodial extracts through Sephadex G-200 column. Significant alterations of erythrocyte osmotic fragility and circulating C-prime levels have been observed in experimental malaria. These changes paralleled the degree of patent parasitemia. The findings indicate development of reliable serodiagnostic procedures is feasible and present methods suitable for following certain immunopathologic responses.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28.	29 OSD CODE	30 BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands)	36
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 114, Malarial antigens

Investigators.
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Associate: Earl H. Fife, Jr., M.S.
Capt Bernard J. Fogel, MC
William A. Hook, Ph.D.
Lawrence E. D'Antonio, D.O.

Description.

This task is concerned with the isolation and characterization of malaria antigens, and the immune response 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 chemical and serologic methods, and (3)development, improvement and evaluation of serologic procedures for detection of antibodies in infected hosts. In vivo studies include (1) the role of antigen and antibody in certain immuno-pathologic responses to 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. Preliminary studies summarized in the previous report (Research Progress Report, 1964) dealt primarily with development of methods for releasing the plasmodia from infected erythrocytes and separating the freed parasites from red cell components. Although saponin appeared to be the best of the lytic agents investigated, extracts from plasmodia obtained in this manner were only moderately reactive in CF tests and were quite anticomplementary. In addition, it was apparent that certain soluble antigen components of the parasites were lost during lysis of the erythrocytes. Efforts to improve the method for collecting the plasmodia and for obtaining serologically active fractions from the parasites therefore were continued.

CO_2-saturated water proved to be superior to any of the lytic agents used thus far for releasing the plasmodia from the erythrocytes. In preliminary experiments, a serologically active antigen fraction was obtained from P. knowlesi as follows: Packed, washed erythrocytes from heavily infected monkeys were lysed with the CO_2-saturated water and the mixture centrifuged. The sediment containing the parasites and red cell stromata was then washed with saline until free from hemoglobin. The sediment was resuspended in saline, homogenized in a tissue grinder, and frozen and thawed several times. The homogenate finally was centrifuged and the clear supernate passed through a column of Sephadex G-200. Successive fractions of the effluent were collected and tested for antigenic activity in CF tests. In addition, each fraction was examined spectrophotometrically and the respective optical densities were used to construct an absorption curve. The single serologically active fraction obtained by this method coincided with the first peak of the curve. Although the fraction reacted well in CF tests with human P. vivax sera, considerable cross reactivity with syphilitic sera was encountered. Furthermore, the antigen was somewhat anticomplementary and was found to be quite labile. Efforts to remove components responsible for the cross reactivity with syphilitic sera by treatment with anhydrous ether completely destroyed the capacity of the antigen to react with malaria antisera. In addition, the antigen did not withstand lyophilization and showed marked reduction of activity even when stored in the frozen state.

Development of more effective methods for separating the plasmodia from red blood cell components resulted in a significant improvement in the quality of the malarial antigen. Methodology based on the differential filtration technic devised by Corradetti (personal communication) resulted in essentially complete separation of the red cell stromata from the parasite material. Antigen was prepared by homogenizing the parasites in a Virtis homogenizer in the presence of glass powder and then centrifuging to remove the gross particles. The supernatant suspension containing the finely divided fragments of plasmodia was frozen and thawed four times and finally clarified by high-speed centrifugation. In certain essential respects, the supernatant antigen was superior to antigens prepared by the previously described methods. The product was essentially free of anticomplementary activity and the cross reactivity with syphilitic sera was significantly reduced. The antigen, however, continued to be quite labile and posed problems of storage. Preliminary analyses indicated that antigens prepared by either of the above methods were primarily protein, and in view of their unusual degree of lability, possibly were lipoprotein in character. Studies to further characterize these antigens are in progress.

In addition to collecting plasmodia from parasitized erythrocytes, efforts are being made to recover additional parasites from the organs of infected animals. The spleen was chosen for initial studies since it characteristically shows gross pathologic changes in acute simian malaria and apparently contains large numbers of parasites. Preliminary results obtained with spleens from Rhesus monkeys with terminal P. knowlesi infection showed considerable promise. Although crude extracts of fragmented spleens showed considerable capacity to react with malarial antisera, they also were somewhat anticomplementary. Passage of the crude extract through a Sephadex G-200 column, however, yielded two serologically active fractions. One fraction reacted well in CF tests with homologous antiserum and apparently contained the major antigen component(s). In addition, the fraction exhibited no anticomplementary activity. The second fraction appeared to contain minor CF antigens but was somewhat anticomplementary. A third fraction of the effluent, which corresponded to a secondary peak of the spectrophotometric absorption curve, exhibited little if any specific serologic activity and contained a relatively high concentration of the anticomplementary components. Efforts to further purify and characterize the major antigen component are being made. Moreover, since the major antigen extracted from infected spleen appears to be quite stable and chemically distinct from those previously obtained from parasites released from circulating erythrocytes, comparative studies from the chemical and serological standpoints will be made.

In conjunction with the above investigations, certain unexpected observations warrant attention since they may provide an insight to some of the pathologic processes associated with simian malaria. It was noted that erythrocytes in the spleens of monkeys with terminal infections contained only early ring forms of the parasite, even though the circulating red cells exhibited a preponderance of mature forms. The spleens also contained many uninfected erythrocytes as well as large numbers of free, mature parasites in various stages of fragmentation. It was further observed that all parasites and red blood cells disappeared when the splenic material was stored for 5 days at 3°C. In view of these findings, cell-free extracts of spleen were combined with parasitized blood and incubated for 18 hours at 37°C in an effort to obtain some insight into the kinetics of this apparent lytic phenomenon. The treatment resulted in complete lysis of both parasites and erythrocytes. Further studies to confirm the presence of hemolytic and/or plasmodialytic factors in splenic tissue are in progress. In addition, similar investigations with other organs, particularly liver, are being considered. Such studies offer interesting possibilities in elucidating some of the mechanisms involved in host-parasite relationships in malaria.

The feasibility of *in vitro* cultivation of plasmodia on a large scale seems remote at the present time. However, in the interests of obtaining malaria parasites in the stage of development most suited for antigen studies, limited investigations on the maturation of parasites *in vitro* are warranted. Preliminary studies along these lines indicated that *in vitro* maturation of the parasites could be induced by placing parasitized blood from a *P. knowlesi* infected monkey in Harvard tissue culture medium containing 25% normal monkey serum and incubating at 37°C. Within 24 hours, the intracellular parasites developed from early ring stages to mature segmented forms. Moreover, it was observed that the merozoites released from ruptured red cells could infect new erythrocytes in the culture and undergo maturation, provided that the initial concentration of parasitized cells was relatively low (*i.e.* < 20%). It is noteworthy, however, that recent experiments have shown that the ability of merozoites to infect erythrocytes was inhibited by malaria antiserum. Studies are being conducted to determine whether this phenomenon can be utilized for detecting malarial antibody..

2. *Studies on the osmotic fragility of parasitized and non-parasitized erythrocytes in experimental malaria.* Results of previous investigations on the osmotic fragility of parasitized erythrocytes in malaria are contradictory. It is the consensus that osmotic fragility of the red cells remains unaltered in malaria (Zuckerman, A., Exper. Parasitol., 15:138, 1964). Nevertheless, studies of some investigators have indicated that the osmotic fragility of parasitized blood cells may be increased. Such findings have been reported by Kondo (Trop. Dis. Bull., 24:658, 1926) in human malaria, by Geiman (J. Exper. Med., 84:583, 1946) in monkeys infected with *P. knowlesi*, and by Danon (Bull. Res. Council Israel, 10E:59, 1962) in mice with *P. vinckei* infections. The first two studies utilized the multiple tube osmotic fragility test whereas the latter employed the "fragilograph" method of Danon. Using the recently developed quantitative technic of Shields and Allen (Department of Hematology, WRAIR), an investigation of the osmotic fragility of erythrocytes in a variety of experimental malaria infections has been initiated. In preliminary studies, a slight increase of osmotic fragility was demonstrated in monkeys infected with *P. knowlesi*. Although the parasitemias obtained thus far have not been of sufficient magnitude to reveal clearcut differences, one monkey with a significant parasitemia (30-40 percent) exhibited a marked increase in osmotic fragility. In addition, it was observed that the parasitized cells could be separated from the uninfected cells by differential sedimentation, and that the osmotic fragility of the parasitized cells (in the top fraction) was significantly greater than that of the non-parasitized cells which constituted the bottom fraction. Studies along these lines are being continued. Moreover they will be extended to include similar investigations on rodent and avian malaria.

3. Complement (C') activity in malaria and other diseases. The observation that in vitro fixation of C' by antigen-antibody aggregates results in a reduction of serum C' activity has led to investigations of total C' levels in a variety of clinical disorders presumed to be associated with abnormal immunologic processes. In humans, reduced C' titers have been reported in patients with malaria (Dulaney, A.D., et al., J. Clin. Invest., 27:30, 1948), acute glomerulonephritis, myasthenia gravis, serum sickness and disseminated lupus erythematosus. The present studies have shown normal or elevated total C' levels in the sera of 6 human prisoner volunteers infected with the Vietnam strain of falciparum malaria. In contrast, similar investigations on the sera of three P. knowlesi infected Rhesus monkeys revealed a significant reduction of hemolytic C' during the terminal phase of acute infection. The decline in serum C' activity moreover appeared to coincide with the period of highest parasitemia, an observation consistent with the reported findings of Roy and Mukerjee (Ann. Biochem. & Exper. Med., 2:245, 1942). Investigations along these lines are being continued and will include malaria infections in other animal species.

Studies on the serum C' levels of 10 patients with acute glomerulonephritis (AGN) and 5 patients with lupus nephritis revealed a marked reduction of C' titers during the acute phase of illness. However, the C' titers of the AGN patients invariably returned to normal levels during convalescence. Although little attention has been given to abnormal elevations of C' activity, it was observed that C' activity was significantly increased in patients with ulcerative colitis and acute rheumatic fever. Moreover, the C' titers generally paralleled the other acute phase reactants (e.g., sedimentation rates, C-reactive protein, etc.) and usually returned to normal levels during the convalescent period. Studies on the serum C' activity in these and a variety of other diseases will be continued as additional requests are received from physicians at the Walter Reed General Hospital and other Army medical facilities.

4. Studies on the possible role of blood group substances in malaria infection. In 1944, Oliver-Gonzalez reported that the titers of anti-A and anti-B blood group substances were elevated in individuals experiencing repeated attacks of falciparum and vivax malaria. Moreover, the titers usually exceeded those observed following a single attack of malaria. Investigations were undertaken to corroborate these findings, and to determine whether P. berghei malaria in rodents was capable of causing an increase of agglutinins to human A substance. Effect of immunization with blood group A substance on the course of P. berghei infection in mice was also studied.

Sera from 6 human volunteers infected with the Vietnam strain of P. falciparum 6 weeks earlier did not contain increased isohemagglutinins. Likewise, mice sacrificed one week after infection with P. berghei showed no increase of anti-A titers. Two Macaca nemistrina monkeys infected with P. knowlesi, however, exhibited a two-fold increase of anti-A agglutinins. Since the P. falciparum and P. berghei infections both were acute in the hosts involved whereas the P. knowlesi infection was chronic, the above findings suggested that anti-A antibody possibly played some role in host resistance. Studies therefore were conducted to determine the effect of anti-A substance on the course of P. berghei infection in mice. In initial experiments, immunization with human A blood group substance in complete Freund's adjuvant elicited demonstrable anti-A titers in all immunized mice. Control animals received either Freund's adjuvant alone, saline, or phenol, the latter being the preservative used in the A substance. The immunized and control animals were challenged with 10^7 parasites 21 days after immunization. The mean survival time for the immunized mice was 12.6 days whereas that for the controls was 8.9 days. Moreover, 82% of the control mice were dead within nine days following challenge. This was in distinct contrast to the immunized group wherein only 14% had expired during this period. In addition, there was a significant delay in the development of patent parasitemia in the immunized animals. Subsequent studies, however, did not corroborate the original observations. It was found that all mice, regardless of whether they had received human A substance, developed anti-A agglutinins approximately 2 months after weaning. The studies therefore necessarily have been delayed until investigations on the ability of mice to develop natural antibodies to human blood group substances could be conducted and a more satisfactory experimental model found.

Summary and Conclusions.

1. A method for obtaining malaria parasites essentially free from contamination with red cell components has been developed. This was accomplished by lysing the infected blood cells with CO_2-saturated water and separating the plasmodia from the red cell stromata by differential filtration. Saline extracts of plasmodia collected in this manner reacted well in complement fixation tests with malaria antisera. The extracts were essentially free from anticomplementary activity and reacted only occasionally with syphilitic sera. Nevertheless, the antigen was quite labile and posed problems of storage. Ancillary studies revealed that the spleens of malaria infected monkeys also were a potential source of parasites; large numbers of free, mature plasmodia were present in the organ. Antigens prepared from these parasites reacted well in CF tests with homologous antibody and in contrast to those obtained from plasmodia harvested from parasitized blood, were quite stable. The spleen also appeared to contain hemolytic and/or plasmodialytic factors, and efforts are being made to determine their possible role as a host defense mechanism. Efforts to induce in vitro maturation of plasmodia in infected erythrocytes were successful.

Moreover, merozoites released from ruptured red cells possessed the capability of infecting new erythrocytes. This phenomenon, however, appeared to be inhibited by malaria antiserum and investigations now are being conducted to determine whether this inhibition has potential value for detecting malaria antibody.

2. Studies on the osmotic fragility of erythrocytes parasitized with P. knowlesi revealed that the fragility of parasitized cells was significantly greater than that of the nonparasitized cells of the same animal. This was most apparent in monkeys with relatively high parasitemias (i.e. 30-40% or higher). To determine whether increased osmotic fragility of parasitized cells may be a common occurrence in malaria and possibly be an important factor in host-parasite relationships in this disease, similar studies are being conducted on P. berghei infections in hamsters and P. gallinaceum in chickens.

3. The effect of human and simian malaria on total complement (C') levels was investigated. Prisoner volunteers infected with the Vietnam strain of P. falciparum showed normal or elevated C' levels. In contrast, Rhesus monkeys with terminal P. knowlesi infections uniformly showed marked reduction of C' activity. Studies to determine whether this reduction is due to a decrease of total C' or due to depletion of one or more individual C' components are being conducted. Individuals with lupus nephritis or acute glomerulonephritis also showed marked reduction of C' levels during the acute phase of illness, but the titers of the latter group returned to normal levels during convalescence.

4. Preliminary studies on various malaria infections suggested that antibody against human blood group A substance possibly plays some role in host resistance. This was further indicated by experiments wherein mice were immunized with A substance and subsequently challenged with P. berghei. The mean survival time of the immunized animals was significantly increased, and the patent parasitemia of these animals appeared considerably later than that of the controls. Development of natural anti-A agglutinins in the mice approximately 2 months after weaning, however, made it difficult to interpret the findings obtained in subsequent experiments with older animals.

Publications.

None.

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 RPT / BRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	4R	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61130011 3A01300191C 01 115

10b PRIOR NUMBER CODE:

11. TITLE: (U) Antimalarial testing in embryonated eggs (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002600 Biology	09 64	NA	OTHER | DA

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

19 GOV'T LAB INSTALLATION ACTIVITY
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ASSOCIATE Davidson, Capt D.E.
TEL 202-576-2280 TYPE DA

21 TECHNOLOGY UTILIZATION: Embryonated eggs

22 COORDINATION: NA

23. KEYWORDS: Plasmodium gallinaceum, malaria, antimalarial drugs, screening

24. (U) Tech Objective - To develop a system for screening of chemical agents for antimalarial activity utilizing embryonated chicken eggs infected with Plasmodium gallinaceum.

25 (U) Approach - Existing techniques for maintaining by intravenous passage the erythrocytic forms of P. gallinaceum in chick embryos have been modified. A reproducible lethal infection has been established against which known antimalarial agents are effective. Effectiveness is evaluated by a prolongation of survival in infected eggs. Test compounds are administered into the allantoic cavity 1 to 3 days post infection.

26 (U) Progress (Sep 64 - Jun 65) - Chloroquine, primaquine, hydroxychloroquine, quinine, camoquine and pyrimethamine are effective in prolonging survival of infected embryos. The effectiveness of 30 additional new agents has been evaluated. The feasibility of utilizing the egg test system for evaluating effectiveness of chemical agents against extraerythrocytic parasites is also being studied.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a. COMSEC OR COMSEC RELATED / X b. NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 115, Antimalarial testing in embryonated eggs.

Investigators.
Principal: David E. Davidson, Jr. Capt VC
Associate: David P. Jacobus, M.D.
David Robinson, Capt VC

Description.

The feasibility of using embryonated chicken eggs infected with Plasmodium gallinaceum as a biological system for screening chemical agents for antimalarial activity has been investigated.

Progress.

The existing techniques for maintaining the erythrocytic forms of P. gallinaceum in chick embryos by intravenous passage have been modified. Thorough washing of the red cell inoculum to remove all anticoagulant reduces early mortality to less than 5%. 10,000 parasitized cells inoculated intravenously into ten to twelve-day embryos produces mortality prior to hatching of 100%, with a mean survival time of 7.9 ± 2.0 days. Inoculation of 2 x 10^6 parasitized cells induces 100% mortality on days 4 to 5. 1.5 or 3.0 x 10^6 cells has been selected as the optimum test inoculation for drug screening since in this range the survival times of individual eggs are sharply restricted to the mean plus or minus one day. Mean survival times of 5.2 days have been observed at this level of inoculum. A variety of known antimalarial agents have been administered into the allantoic cavity of infected eggs 1 to 3 days post inoculation, and are effective in producing significant prolongation of survival time.

Summary and Conclusions.

Chloroquine, primaquine, hydroxychloroquine, quinine, camoquine and pyrimethamine are effective in prolonging survival of chick embryos lethally infected with P. gallinaceum. Thirty new agents gave test results comparable with rodent test results. In general chemical agents are more toxic in chick embryos than in mice.

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RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6498	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 116

10b PRIOR NUMBER/ CODE: None

11. TITLE: (U) Experimental search, retrieval, and printing of Biological Abstracts (09: 39)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002600 Biology; 004200 Computers	02 65	NA	OTHER / DA

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

19 GOV'T LAB INSTALLATION ACTIVITY
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INVESTIGATORS PRINCIPAL ASSOCIATE: Jacobus, Dr. D.P.
Gulick, R.
TEL: 202-576-2280 TYPE: DA

21 TECHNOLOGY UTILIZATION: Universities; pharmaceutical industry; chemical industry

22 COORDINATION: NA

23. KEYWORDS: Abstracting and indexing; biological sciences; communication, information processing; information retrieval, libraries; medicine in literature

24. (U) Tech Objective - To provide abstract services for WRAIR Investigators to determine "literatureness" of abstracts; and to improve reader use with special attention to developing techniques so that effective systems can be designed; to provide measurable information on the future role of abstracts and assist in development of techniques for manipulation of literature.

25 (U) Approach - Biological Abstracts provides coverage of approximately 100,000 abstracts per year. Machine tapes for the index terms, author, original citations and microfilm of 500,000 abstracts are available. WRAIR personnel request abstracts by author, selected words, subjects, or broad areas of interest. These bits of information are entered into the computer, with a printout provided of all abstracts pertaining to the user's request. Hard copies of these abstracts are provided the requestor within 24 hours.

(U) Progress - (Feb 65 - Jun 65) System became operational on 1 June 1965.

26

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY a COMSEC OR COMSEC RELATED / b NOT RELATED	28	29 OSD CODE	30 BUDGET CODE
X		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) FY ...	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 116, Experimental search, retrieval, and printing of Biological Abstracts

Investigators.
Principal: David P. Jacobus, M.D.
Associate: Mrs. Mary F. Jackson

Description:

An experimental project with Biological Abstracts is outlined. This project has three main objectives: first, to provide abstract services for the US Army Medical R&D Command capable of supporting not only WRAIR but also the overseas laboratories; secondly, to experiment with the "literatureness" of abstracts, and thirdly to improve reader use with special attention to developing measurement techniques so that attractive and effective systems can be designed. Biological Abstracts have been selected for this experiment because they constituted the smallest body of literature of interest to WRAIR with which a meaningful experiment could be done, and they play a very real role in the manipulation and use of the published literature. Their future role has been under discussion. The experiment outlined here may give some measurable information on their future role and assist in the development of techniques for the manipulation of literature.

In the first area of experimentation, namely the provision of abstract services for the U.S. Army Medical Research and Development Command, one of the key problems faced by anyone attempting to use literature is the fact that there is no single source for all the information. By virtue of the fact that there is no single source, the user desiring complete coverage must, of necessity, check all additional sources. It is therefore important that if an abstract service is to be provided that the service cover completely a relatively large and well-defined block of literature. In this experimental service, it is planned to cover all the Biological Abstracts back to Volume 58 published in 1959. This will provide coverage on a half-million abstracts of good quality and relatively good coverage of tropical diseases, and infectious parasitic disease. One of the purposes of this abstract service experiment is to reduce the penalty for using an abstract journal, namely, to provide the individual user with a collection of abstracts that he has inspected so that there is no need to make notes on the abstracts as they are read, spend the time looking up each individual abstract or the time copying them if they are to be appended to the draft of an experimental protocol. The last purpose of this abstract experiment is to supply on demand to the overseas WRAIR laboratories and other portions of the R&D Command a quick series of abstracts in response to queries. There are times when WRAIR personnel are not supported with respect to local library

facilities. The prompt shipment of abstracts will provide field investigators with library back-up. This aspect of library support will justify in itself the expense for microfilming and printing out the information in response to queries.

Secondary experimentation deals with the "literatureness" of abstracts. When abstracts were originally devised, the number of articles appearing in the open literature were relatively few. The abstracts therefore served as an alerting device so that all workers in the field could read the abstract and decide whether they desired to see the original citation. In view of the massive growth of literature, abstracts are no longer functioning as alerting devices. Instead the alerting devices are indices and permuted titles although some experimentation with the selective dissemination of abstracts still exists, so in this case abstracts are still serving as alerting oevices. The dissemination procedures usually fall into two broad classes.One is a relatively coarse selective scheme in which major abstract journals are published in separate sections. For example the separate sections in which Biological Abstracts used to be published are comparable to the broad sections in which Chemical Abstracts are now published. The second broad division is a relatively specific selection and dissemination of abstracts such as the experiments carried out by H.P. Luhn or the literature services offered by the Library of Congress or the Office of Technical Services in the Department of Commerce. Both of these selective dissemination procedures provide abstracts on a regular basis, rather than in response to a specific inquiry at the time an investigator asks a question. Given abstracts are becoming less effective as alerting devices: there is a question as to their future role since the alerting function is no longer paramount. On the other hand as their alerting function decreases they serve an increasing role as literature. First they occupy a permanent place in the library. Secondly, they often serve as definitive literature in the negative sense, namely, when a series of index terms are looked up leading to abstracts, the researcher in the library will often read the abstracts and then decide that he no longer desires to look at the final article even though the article is on an adjacent shelf. In this sense the abstract has served as a definitive reference. There have never been any measurements as to how often an abstract serves as a definitive reference, yet in this negative sense, such measurement standards are necessary if we are to develop some insight into the effectiveness of our index systems and index manipulation services. Abstracts also serve as literature in that a collection of abstracts on a single specific subject in response to a query can brief an investigator on the coverage of the broad outlines of the subject matter in question. Such a collection of abstracts on a specific subject constituting a review of a subject also serves as literature.

The third area of experimentation is the attempt to develop improved reader use and to develop experimental procedures for increasing reader use and measuring the results of such manipulations. The number of people doing research is far greater than the number who know how to use a library effectively. As a result, research is often done on subjects already well understood or in areas that are only marginally novel. The people capable of using libraries use abstracts as a major tool, yet if one inspects the traffic pattern and reader pattern in a technical library, many people come in, browse

through the current issue room without making a regular attempt to be certain of complete coverage by looking in the abstracts. The ease of browsing through some of the current issues undoubtedly accounts for part of this pattern. Ignorance of the role of abstracts and the difficulty of using them accounts for some of the differences in use. Other differences are the likelihood of finding interesting information by virtue of the specialization of the journals, the possibilities of obtaining reprints from journals (but not from abstracts) so as to augment private files, the opportunity to read in detail immediately rather than hunt for the original reference. With some abstract journals, for example, Biological Abstracts and Nuclear Science Abstracts, the delay between the appearance of the original article and the appearance of the abstract journal is remarkably short, e.g. one month to six weeks in the case of Biological Abstracts. The delay in publishing these journals or delay in indexing is probably not the paramount feature in their lack of use.

Part of the excitement in information retrieval lies in the possibility that computer manipulation of requests for information and of the information itself will provide better services. Such services will only be available if they can be presented in such a way that investigators will use them. Such presentation requires consideration of relatively subtle human factors. Some information services are pretty good now but they are not much used. A crucial portion of this experiment is therefore to experiment using the existing literature with new techniques designed to induce users to use the information. Crucial to the understanding of these experiments on increasing reader use is the understanding that none of these experiments on utilization depend on the re-education of users in attempting to persuade users to think in new patterns, to learn new index terms or to deviate from their existing habits. Users in general are busy and have an instinctive ability to avoid a prolonged literature search in which the rate of retrieval of non-pertinent information is relatively high. They can very quickly thumb through a small collection of abstracts in response to a specific query and screen out the ones that are of no interest and in fact will do so. This quick browsing is part of the manner in which they go through the open reading room.

The initial plan to improve reader use involves the availability of equipment on an open basis, namely, one which will enable an investigator to use the equipment quickly without any special instructions and to receive a prompt answer in the form of hard copy. Such use is established with the hope that the attractiveness and advantages of the reading room open stack system can be duplicated by making these new mechanical manipulations on an "open stack" basis. By placing the input devices at strategic locations within the library it may be possible to measure what specific criteria are necessary to insure reader attractiveness. For example, an inquiry station like an electrowriter near Biological Abstracts would permit a user to look up in an index an appropriate subject reference, then turn to the abstract journal and look up the abstracts in question. The reader would then know that the subject reference was in an area in which

he was interested. He could then obtain a permanent record by writing the request and subject reference on the electrowriter, or he could write the specific abstract and volume numbers on the electrowriter. In either case he would receive a hard copy answer to his questions. As a future development a similar inquiry station near a subject index of Biological Abstracts (for example, in the halls in the same manner that a telephone booth is located in the hall) could measure the need of a reader to check immediately pertinence of the subject headings by looking at the appropriate abstracts. Such an experiment would also provide some data on the necessity of printing a complete set of abstracts or just printing an abstract in response to an inquiry. A control for such a peripheral inquiry station using a subject index would be another inquiry station such as above but without the subject index, so that the questioner would just ask the question or the subject term without reference to the existing file. This inquiry station would provide information as to the need for publishing a subject index or a thesaurus. It would also provide some information as to whether users would use an inquiry station outside of the library more frequently if they did not have to walk down one floor. Another inquiry station might be located in the reading room, also without a subject index being nearby, so that if a reader were to develop an interest in a subject he could immediately ask for it on the electrowriter without going through the difficulty of looking up specific citations. Based on vast experience which has occurred since 1900 on the advantages and increased reader attractiveness of the open stack system versus the closed stack procedures the main burden of these experiments is to increase user attractiveness. These experiments look forward to developing techniques that will permit researchers without specialized training to use the modern manipulative procedures for information handling without the necessary intervention of librarians. The burden of flexibility of use is laid upon the development of specialized hardware and computer programs which will meet the requirements of the user rather than re-educating the users to use special techniques. The inquiry station without any subject matter associated with it will also provide valuable information on the manipulation procedures necessary to support inquiries from personnel operating where there are absolutely no library facilities.
A considerable foundation for these reader use experiments is also based on experience of the telephone company with user patterns. There are two fundamental experiments that are worth citing in support of using multiple input stations: The telephone company in its long lines division used to operate a wonderful service which now is available on an optional basis. This service involved the user calling an operator saying that they would like to ring a number in a distant city. The operator would then place the call and inform the user if the distant number were busy, etc. If it were busy then the caller could ask the operator to call again in 20 minutes or to try the call in an hour if the caller desired to step out. The introduction of distant dialing gave callers an option, namely, they could still call the operator or they could place the call themselves, even though this meant looking up additional numbers. By placing the call themselves the users can obtain slightly faster service, but they would not obtain the benefits of having the operator try the number again in case of a busy circuit. The introduction of this dial through service resulted in a 25 percent increase in the number of long distance calls. The system, when first introduced, required that an operator intervene during the placement of the call in order to determine the calling number for purposes of billing. The introduction of automatic billing equipment so that the entire process was automatic resulted in an

increase of over 50 per cent of calls calculated over and above the improved rate resulting from the first introduction of direct distance dialing. The second broad area of experiments from the telephone company lies in their well-established observations that an increased number of phones at convenient locations results in an increased volume of business. Therefore, the development of such automatic dial through procedures is of paramount importance in the improvement of reader use of library facilities. Costs of such cheap convenient, easily used input-output devices (as electrowriters) are minimal in comparison to the costs of doing research in an 8 million dollar facility without active literature utilization.

Other methods of information handling have resulted in the development of new computer tools for manipulation of index terms. Since the index terms constitute the primary process by which investigators are alerted to the current literature, the development of easy techniques for the manipulation of these index terms is of paramount importance. For an understanding of how these procedures operate, one must understand that the manipulations which have been successful to date for the improvement of indexing of articles and the retrieval of pertinent information all depend upon very fast feedback and dialogue between the reader and the questions and the answers. For example, in the current system as it is now practiced, if a reader looks up an index term and finds a long list of subheads under this term, he then is inclined to pick out the subheads which are most pertinent with respect to his question. Such selection is based on the past "feedback" resulting from his new awareness of the size of the list of subheads. If he then looks up the abstracts pertinent to this subhead and finds that the information is really not useful he then goes back and may perhaps try "a see also reference." One of the problems in the development of an open machine stack system using computer techniques is the difficulty of obtaining feedback in response to a question. Such feedback is important if the reader is to mature in insight. Time shared computer systems permitting dialogues are now becoming feasible but need further study. A major system in existence is the system at MIT, Project Mac. Different systems permit the opportunity of a prompt response. Examples of the advantages of response so that the user can further refine his questions (without enjoying the flexibility of machine manipulation procedures) are a printed index or a thesaurus. The importance of feedback has also been demonstrated by the manipulation of index terms as published by Stiles: Stiles' manipulation which was originally done to improve poor indexing depends on the reader being able to look over the suggested terms and re-ask the question. The reader must therefore receive the feedback sufficiently quickly that the additional questions can be asked while the reader is still interested in the subject.

This speed of response necessary to satisfy a reader is the subject often not appreciated. If a reader has to wait an appreciable length of time to obtain a reference, unless he is indulging in a literature survey after the experimental details have been completed, he will tend not to wait for the information but rather will reason around it. The most important period of retrieval, therefore, lies in the formulative period when new projects are being developed, when the reader is actively indulging in a dialogue with references which immediately come to hand. Considering the large amounts of

technical information and complex decisions which an investigator must make in the limited period of time available, immediacy of coverage such as provided by the immediate provision of abstracts obtainable by looking up a subject term or the browsing through the open reading room is necessary.

Costs: An automated information service using inquiries into a computer system has been attempted in the past. In general such services have imposed a penalty upon the user who is charged for the inquiries. This additional cost penalty is quite different from the penalty which a user usually pays with respect to the open literature in a library, namely, the library subscribes to the journal for a given fee after which the journal is available for anybody to use. In general only one person can use a journal at one time, but the journal is on the shelf and constitutes an inquiry station. The present cost arrangements visualized in this experiment would be to consider each input electrowriter as an inquiry station which would be subscribed to in the same manner that one subscribes to a set of Biological Abstracts. This inquiry station could then be used free by all members of the subscribing institution. In general, if the technical personnel were required to pay each time they opened a journal the poor utilization of the existing library facilities would be enhanced. This same use now-pay later concept is also evident in other areas of the economy. It is most important to introduce this concept into mechanized library use in order to establish that people will continue to be encouraged to use the library.

Progress:

The system has been in effect for approximately 3 weeks. There have been approximately 100 requests which involve the searching and the printing of 7,000 abstracts if one word was used in the question. If one word plus one index term from the cross index system is used in a question, 1,000 abstracts are searched for approximately every abstract printed for distribution to the submitter. For example, on a question dealing with iron metabolism 5,500 abstracts were searched for 7 abstracts printed. The system appears to be popular with library users. Further experience will be necessary.

Summary:

A new inhouse project with Biological Abstracts has been initiated which has three objectives. The first objective is the provision of abstract services for the WRAIR. These abstract services are designed to provide a coverage on a sizeable block of open literature. It therefore differs from abstract services offered by other Department of Defense projects by virtue of its emphasis on open literature and its emphasis on biology. The second area of the project deals with the evaluation of "the literatureness" of abstracts since the definitive measurements on this subject have not been made and are of key importance, if mechanistic services are ever going to be installed. The third area of the project is an attempt to prove the overall ability of

investigators in the Walter Reed Army Inst. of Research to use literature. Associated with these three endeavors are some ancillary projects involving new techniques in machine manipulation of index terms and indexing techniques This project has been functioning for approximately three weeks at the time this report is written. Preliminary information indicates that meaningful information will be gained in all three areas. The popular reception of the technique within the WRAIR appears to be favorable.

Conclusions:

In the first area of experimentation it appears that the provision of abstracts from the open biological services satisfies a need within the WRAIR. A considerable number of questions have been asked. Answers have been provided which have been useful in the conduct of research. In the second area of experimentation (the literatureness of abstracts) it is apparent that abstracts do constitute literature more than 50% of the time. It is apparent from the naivete of the questions that considerable work either through education or improved computer programing will be necessary.

Publications:

None

RESEARCH AND TECHNOLOGY RESUME

1.	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6499	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	JR	A. WORK UNIT

10a CURRENT NUMBER/CODE	10b PRIOR NUMBER CODE
61130011 3A013001910 01 117	None

11 TITLE
(U) Problems in Algebra (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
012100 Organic chemistry 009700 Mathematics & statistics	06 65	NA	OTHER / DA

16 PROCURE METHOD	17 CONTRACT GRANT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
A. GRANT	a DATE 06 65 b NUMBER DA 49-193-MD-65G-177 c TYPE Y. GRANT d AMOUNT $3912	PRIOR FY CURRENT FY		

19 GOV'T LAB INSTALLATION/ACTIVITY	20 PERFORMING ORGANIZATION
NAME Headquarters ADDRESS U.S. Army Medical Res & Dev Command Washington, D.C. 20315 RESP INDIV Tigertt, Col. W.D. TEL 202-576-3551	NAME University of Maryland ADDRESS College Park, Maryland INVESTIGATORS PRINCIPAL ASSOCIATE Pearl, M.H. TEL 301-WA 7-3800 TYPE A

21 TECHNOLOGY UTILIZATION	22 COORDINATION
Algebra, and matrix conversion	NA

23 KEYWORDS
Algebra, linear equations, matrix conversion

24. (U) Tech Objective - To assign values to substituents on closely related chemical series to guide the synthesis program and assist in the correlation of chemical structures versus biological function.

25 (U) Approach - To evaluate the system of over-determined linear equations and subsequent matrix conversion proposed by Free and Wilson in order to determine techniques for handling relatively large systems.

26 (U) Progress - New.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) FY	36

DD FORM 1498 Items 1 to 36 identical to NASA Form 1125 REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6500	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT / WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U / U	NA	JR	A. WORK UNIT

10a CURRENT NUMBER CODE: 61130011 3A013001A91C 01 118

10b PRIOR NUMBER CODE: None

11. TITLE: (U)Electron microscopy of intestinal epithelium (33)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
003500 Clinical Medicine	02 65	01 66	OTHER / DA

16 PROCURE. METHOD	17 CONTRACT GRANT	18 RESOURCES EST	a PROFESSIONAL MAN YEARS	b FUNDS (In thousands)
B. CONTRACT	a DATE 02 65 b NUMBER DA 49-193- MD-2705 c TYPE A. FPF d AMOUNT 26,424	PRIOR FY CURRENT FY		

19 GOV'T LAB INSTALLATION ACTIVITY
NAME: Headquarters
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TEL: 202-576-3551

20 PERFORMING ORGANIZATION
NAME:
ADDRESS: New York Medical College
5th Ave at 106th St
New York, New York 10029
INVESTIGATORS PRINCIPAL ASSOCIATE: Hartman, Dr. R.E.
TEL: 212-TRafalgar 6-5500 TYPE: B

21 TECHNOLOGY UTILIZATION: Medical research

22 COORDINATION: NA

23. KEYWORDS: Hepatitis; sprue; electron microscope; virus; intestine

24.
(U) Tech Objectives - To characterize the intestinal lesions of tropical sprue and infectious hepatitis. To attempt to identify virus particles in specimens from patients with these diseases.

25 (U) Approach - By electron microscopic examination, intestinal specimens from U.S. soldiers with infectious hepatitis and tropical sprue are being studied to show the changes that occur, relate them to physiologic abnormalities and attempt to demonstrate viral particles.

26 (U) Progress (Feb 65 - Jun 65) - Intestinal specimens have been prepared for electron microscopic examination from patients with tropical sprue in Puerto Rico and soldiers with infectious hepatitis in Korea. A stellate shaped body composed of 20 mu aggregates has been identified in the jejunal epithelial cells of patients with infectious hepatitis. Further study by a variety of techniques is necessary to ascertain if these are virus particles.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) CFY+1	36

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 118, Electron microscopic study of certain aspects of the intestinal epithelium in infectious hepatitis, tropical sprue and related conditions

Investigators.
Principal: Roberta Hartman, Ph.D. (New York Medical College)
Associate: Lt. Colonel Thomas A. Sheehy, MC, and Major Marcel E. Conrad, MC

Description.

A collaborative project to investigate the electron microscope changes in intestinal specimens from patients with infectious hepatitis and tropical sprue.

Progress.

Previous collaborative endeavors have shown the electron microscopic intestinal changes in specimens from patients with tropical sprue. The alterations observed in the microvilli were believed to cause the abnormal absorption of sugars and fat from the diet. Further studies are necessary to document changes within the intestinal mucosal cell and to attempt to demonstrate a possible virus in these specimens. Material is obtained from U. S. soldiers returning from Puerto Rico and Southeast Asia with tropical sprue.

Recent studies have demonstrated light microscopic alterations of intestinal histology in specimens from patients with infectious hepatitis and certain other viral illnesses. Since the gut is the organ of entry for hepatitis and feces is infective, it was thought that the virus might be identified in electron micrographs of intestinal mucosal cells. Most studies of hepatitis have examined alterations in the liver. It is possible that hepatitis -- similar to poliomyelitis -- may cause a commonplace intestinal infection while involvement of other organs is sparce and infrequent. Preliminary examination of small intestinal biopsy specimens has shown a stellate shaped body within the cytoplasm of intestinal mucosal cells of patients with infectious hepatitis. These structures are similar in size, shape and configuration to aggregates observed by Taylor, et al., in the liver of patients with infectious hepatitis and which he believes to be the causative organism of this disease. Further studies are necessary to investigate these observations and speculations. Specimens are available from documented cases of hepatitis in U. S. soldiers.

Summary and Conclusions.

Intestinal specimens from U. S. soldiers with tropical sprue and infectious hepatitis are available for examination by electron microscopy. Alterations in intestinal histology are being studied and attempts are being made to try to identify the causative agents of these diseases.

Publications.

Conrad, M. E., Hartman, R. and Astaldi, G.: The gastrointestinal lesion in infectious hepatitis. Ann. Int. Med. 60: 723, 1964.

Sheehy, T. W., Artenstein, M. S. and Green, R. W.: Small intestinal mucosa in certain viral diseases. J.A.M.A. 190: 1023-1028, 1964.

Conrad, M. E., Schwartz, F. D. and Young, A. A.: Infectious hepatitis - a generalized disease. A study of renal, gastro-intestinal and hematologic abnormalities. Amer. J. Med. 37: 789, 1964.

RESEARCH AND TECHNOLOGY RESUME

1.	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6501	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT	6. SECURITY WRK	7 REGRADING	8. RELEASE LIMITATION	9 LEVEL OF RESUME
30 06 65	A. NEW	U	U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 01 119

10b PRIOR NUMBER CODE: None

11. TITLE: (U) Cytochemical Analysis of Growth of Malarial Parasites (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUND'G AGENCY
002300 Biochemistry 010100 Microbiology	06 65	NA	OTHER DA

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

19 GOV'T LAB INSTALLATION ACTIVITY
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TEL: 202-576-3551

20 PERFORMING ORGANIZATION
NAME: Armed Forces Institute of Pathology
ADDRESS: Walter Reed Army Medical Center
Washington, D. C. 20012
INVESTIGATORS PRINCIPAL ASSOCIATE: Bahr, Gunter F.
TEL: 202-576-2915 TYPE: DA

21 TECHNOLOGY UTILIZATION: Biochemistry, medicine

22 COORDINATION: NA

23 KEYWORDS: Malaria; Plasmodium; Metabolism; Microscopy; Cytology

24. (U) Tech Objective - To determine the quantitative aspects of growth of malaria parasites in terms of rate of synthesis of proteins, lipids and nucleic acids.

25 (U) Approach - To examine the cellular and subcellular entities comprizing malaria parasites by cytospectrophotometry, interference microscopy and quantitative electron microscopy.

(U) Progress - New.

26

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

31 MISSION OBJECTIVE	32 PARTICIPATION
	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT
NA	

35 EST FUNDS (In thousands) FY 71	36

DD FORM 1 AUG 68 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01
Biochemistry

RESEARCH AND TECHNOLOGY RESUME

1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6430	CSCRD-103

4 DATE OF RESUME	5 KIND OF RESUME	6 SECURITY RPT	6 SECURITY WRK	7 REGRADING	8 RELEASE LIMITATION	9 LEVEL OF RESUME
01 07 65	A. NEW	U	U	NA	JR	A. WORK UNIT

10a CURRENT NUMBER/CODE: 61145011 3A014501B71P 01 070

10b PRIOR NUMBER CODE: NONE

11 TITLE: (U) Biochemical activity in health and disease (09)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
012900 Physiology; 002300 Biochemistry	06 62	NA	OTHER | DA

16 PROCURE METHOD	17 CONTRACT GRANT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
C. IN-HOUSE	c TYPE NA	PRIOR FY 65	11	180
		CURRENT FY 66	9	140

19 GOV'T LAB INSTALLATION ACTIVITY
NAME: Headquarters
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Washington, D. C. 20315
REM. INDIV: Cutting, Maj R.T.
TEL: 202-OX 64458

20 PERFORMING ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D. C. 20012
INVESTIGATORS PRINCIPAL: Knoblock, Col E.C.
ASSOCIATE: Mertz, W. M.D.
TEL: 202-576-3528 TYPE: DA

21 TECHNOLOGY UTILIZATION: Biochemistry; Physiology

22 COORDINATION: NA

23 KEYWORDS: Motion sickness; labyrinth; vestibular stimulation; fatty acids; enzymes; enterotoxin; enterobacteriaceae; catecholamines; histamine; nucleoproteins

24 (U) Tech Objective - The basic biochemical responses to injury and disease will be studied in order to provide more definitive information for the clinical management or prevention of disease or disability.

(U) Approach - The modern tools of biochemistry are brought to bear on both physiological and biochemical studies of diseases and disorders.

25

(U) Progress - (Jul 64 - Jun 65) - A biochemical study of motion sickness showed changes in enzyme levels, acid-base balance, and glucose utilization attending nausea in men with normal labyrinth function. Deaf subjects (labyrinth deficient), did not show these responses. A study of the toxin-producing capacity of enterobacteriaceae indicates that lipids are required for endotoxin synthesis and that glucose reduces synthesis of Kreb's cycle enzymes in these species. Dietary trans fatty acids were found to be incorporated into rat depot fat and membranes and were shown to alter membrane functions. Using countercurrent distribution procedures, three serine
26 transfer-RNAs from yeast were separated and two were purified. Additional work with this technique has shown that the procedure is capable of separating oligonucleotides that vary only in their nucleotide sequences.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
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35 EST. FUNDS (In thousands) CY++	36

DD FORM 1 AUG 64 1498 (Items 1 to 36 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

Investigators.

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Description: Various disciplines have been brought to bear on a series of medical problems with emphasis on biochemical characterization of responses to disease or further detailed knowledge of mechanisms relating to development or treatment of disease. In development of this program it was intended that talent available would be extended outside the WRAIR whenever required to study biochemical processes important to military operations. A field study regarding mechanisms of motion sickness has resulted from this capability as have other studies reported elsewhere in this report.

Progress:

Biochemical Responses to Vestibular Stimulation - This study was an extension of a previous study (1962) in which it was observed that pilots maintained in a constantly rotating room at the Naval School of Aviation Medicine, Pensacola, Florida, not only developed an acute motion sickness but reported an unusual degree of fatigue. It was observed that the efficiency of these men was markedly reduced after a short period of time in the room.

The biochemistry studies were designed to determine not only the physiological changes but also to determine the involvement of the labyrinth complex in producing the syndrome. Two groups were utilized for a two-week period each. The first group consisted of four pilots who were normal in all respects. The second group were selected as labyrinth deficient (LD), but normal otherwise.

Biochemical examinations included acid-base and electrolyte studies, intravenous glucose tolerance, complete urinalysis and blood counts, serum enzyme and isoenzyme studies.

The normal group showed the characteristic response of motion sickness within a few hours of beginning rotation. This continued for approximately 48 hours at which time they resumed eating; however, their alertness and ability to function properly as pilots were reduced. The LD group did not show any of these effects.

Of the biochemical parameters measured, the most striking finding in the study was the demonstration of an increased glucose utilization by normal subjects in comparison with the LD group. These findings are consistent with a hypothesis drawn from the previous study (pilots '62) that changes in glucose tolerance may be caused by increased muscle tonus in normal subjects. The increase in tonus may be a direct result of labyrinth stimulation. This hypothesis is supported by determination of pH which showed a decrease; by an increased pCO_2, and by a steady or slightly decreased pO_2 value for normal subjects. The LDH value was also increased in the pilot group only. These are the type responses that would be expected. The LD group should not show such a response. With a possible exception to an unexplained response in pO_2 in the pilot '64 group, this hypothesis is borne out. An additional definitive experiment with especially careful control of diet, work regimen, and subjective observations is indicated to further assess the differences between the responses of normal and labyrinth-deficient men when subjected to vestibular stimulation.

Effects of Trans Isomers of Unsaturated Fatty Acids in Membranes - Since trans fatty acids are present in oleomargarines and therefore consumed by a large segment of the American population, it was decided to study the incorporation of these compounds into the tissues of an experimental animal, the laboratory rat, and the resultant effects upon the animal at the membrane level. Erythrocytes and liver mitochondria were selected as representatives of cellular and subcellular membranes, respectively.

Weanling male rats were fed diets supplemented with, and deficient in, trans fatty acids for a period of six (6) weeks. Blood, liver, and epididymal fat pads were removed. No significant differences were noted in relative weight gains of animals fed the two diets, nor were any gross pathologic lesions evident at sacrifice.

Individual fatty acid concentrations were determined in erythrocyte stroma, liver mitochondria, and depot fat. There was considerable incorporation of a trans fatty acid, elaidic acid, into all three tissues.

Dietary trans fatty acids appeared to influence the distribution of other fatty acids, both cis isomers and saturated fatty acids; rats fed a trans supplemented diet had a lower concentration of saturated isomers than did the control animals. Total content of trans fatty acids in the three tissues were studied; the content of these compounds in depot fat was closer to that of dietary lipid than was the trans isomer level of the two structural lipids.

Swelling rates of mitochondria from rats fed trans fatty acids were from two to three times as rapid as the rates of controls. Erythrocytes from rats fed the high-trans diet were more resistant to osmotic hemolysis than were the cells from control animals while erythrocytes from animals fed trans fatty acids were more susceptible to lecithinase-induced hemolysis. Erythrocytes containing large amounts of trans isomers were more resistant to deoxycholate-induced hemolysis. No difference was noted between responses of the two dietary groups to metal-induced hemolysis. Erythrocytes from rats fed trans fatty acids showed a slightly increased permeability to glycerol, but not to diethylene glycol, nor to thiourea. Erythrocytes from rats fed high trans isomer levels actively transported sodium ion into the extra-cellular medium at a slightly faster rate than did the control cells.

Biochemistry and Genetics of the Enterobacteriaceae - The work on the biochemistry and genetics of the Enterobacteriaceae centered around four main areas of investigation:

a. Mode of Action of Endotoxin. The fatty acid make-up of endotoxin from Serratia marcescens grown under different temperatures and media have been studied. It has been established that varying growth conditions do not appreciably affect the potency of endotoxin preparations but do greatly affect the quantity per cell. This is supported by the recent work of Horecker's group showing the requirement of lipid for endotoxin synthesis. Umbreit has shown that biotin-deficient mutants do not synthesize the lipopolysaccharides of the cell wall. It is planned to look for these biotin mutants and discover what the endotoxin potential is of these cells, since Corwin and Farrar have postulated that lipid is required for endotoxin potency.

Guinea pigs have been treated with sub-lethal amounts of a variety of agents causing liver damage: carbon tetrachloride, allyl alcohol, and ethionine. Although homogenates from all livers were unimpaired in their ability to oxidize succinate, there were great differences in the ability to oxidize octanoic acid. There was excellent

correlation between the lowered ability to oxidize octanoic acid and the animal's ability to withstand endotoxin. This supports our previous evidence that the liver enzymes which inactivate endotoxin are related to fatty acid oxidation.

b. Genetics of Kreb's Cycle Enzymes. To date, mutants of E. coli have been isolated which lack isocitric dehydrogenase (ICD) and phosphoenol pyruvate (PEP) carboxylase. A third mutant grows poorly on glucose alone but normally when α-ketoglutarate, succinate, or malate are added as growth factors. It does not grow on acetate even with added growth factors. Enzyme analyses of this mutant reveal that aconitase (measured by TPN reduction at 340 mu) is low although not absent. When aconitase is measured by measuring aconitate formation directly at 240 mu, the aconitase activity is not decreased in the mutant. This discrepancy has been traced to a very high level of TPN oxidase in the mutant. This depression of the enzyme is probably due to an accumulation of TPNH. Since this mutant is also unable to ferment arabinose, investigation is proceeding to determine the possibility of a block in the hexose monophosphate shunt around ribulose-5-P.

c. Biochemical Control Mechanisms of the Kreb's Cycle. Studies of the effect of the content of the medium on growth and level of Kreb's cycle enzymes were initiated upon the discovery that although growth of E. coli is greater on Brain-Heart-Infusion broth (BHI) than on a meat extract broth (MEB) the Kreb's cycle enzymes were severely depressed. This was traced to the presence of glucose as a carbon source in BHI which is absent in MEB, by verifying this effect in minimal media with glucose as a carbon source on the one hand and either acetate or a Kreb's cycle intermediate on the other. The latter group induces very high levels of Kreb's cycle enzymes and glucose represses to low levels. Apparently there are also great differences when the bacteria are taken from lag phase as compared with the resting state. When similar studies have been made with PEP carboxylase, it was found that this enzyme is induced when glucose is the carbon source and repressed when Kreb's cycle intermediates are used. The possibility was considered that glucose stimulated PEP carboxylase, thereby increasing the amount of oxaloacetate (OAA) and the OAA repressed enzymes such as malic and succinic dehydrogenases. This hypothesis was tentatively rejected when our PEP carboxylase mutant which lacks the enzyme still showed glucose repression of these two enzymes.

d. Mechanism of Penetration of Virulent Shigella into Host Cell. Collaborative studies of the mechanism of penetration of virulent Shigella flexneri have been undertaken. Use is being made of a naturally occurring colonial mutant which is avirulent to compare activities of enzymes suspected of contributing to the penetration. It has been established that the permeability of a variety of substrates is considerably lower in the avirulent strain as determined by oxidation studies before and after sonication. It was thus hypothesized that the bacteria penetrates by some enzyme action on the host cell wall. In the avirulent strain the substrate in the cell wall cannot penetrate to the enzyme. We have labeled Hela walls with glucose-U^{-14} C and incubated these labeled cell walls with the bacteria but were unable to distinguish any consistent differences in production of CO_2 and soluble sugars. We are also trying to ascertain whether pinocytosis by the Hela cell is in part responsible for the bacteria appearing in the cell.

Studies of Chemical Structures of Nucleic Acids - The role of transfer-RNA in protein biosynthesis in the transfer of the activated specific amino acid to the site of protein synthesis is being studied. The existence of multiple transfer-RNAs corresponding to single amino acid has been shown to exist. These multiple s-RNA forms with a single specificity have been shown to have differential interspecies specificity and also different code recognition sites on messenger RNA.

Using countercurrent distribution procedures, three serine specific transfer RNAs were observed to exist in yeast. Two of these three RNAs were purified. Some of their biological and chemical differences and similarities include observation that the rate and extent of serine incorporation by both yeast and rat liver amino acyl s-RNA synthetases are quite similar. Both of the RNAs are inactive, with E. coli enzymes. The in vitro binding of charged s-RNAs to ribosomes, responds equally to UpCpUp and UpUpC.

The two RNAs were subjected to pancreatic RNase and mapped in order to determine the resulting mono and oligonucleotide contents. Sixteen different components were identified. Out of these, Ser. II RNA contained 1 mole of (ApCCp) CCpUp and two moles of ApUp, whereas Ser. III RNA contained two moles of (ApCCp) CCpUp and one mole of ApUp. The remainder of the components were essentially the same in both RNAs. The analysis of the components obtained with RNase T. digestion of these two RNAs is currently in progress. In order to extend these studies for the complete elucidation of the chemical structures of these RNAs, the following projects were carried out to

develop much-needed methods. Yeast s-RNA was subjected to pancreatic RNase and the degradation products were fractionated by chromatography on DEAE-cellulose in 7 M urea, with a NaCl gradient. Fractions corresponding to hexa and heptanucleotides were further fractionated by 700 and 400-transfer countercurrent distribution, respectively. Two fractions present in the hexanucleotide appear to be $(Ap)_2 (CCp)_4 Cp$, $(Ap)_4 (CGp)_2 Cp$, and a nonanucleotide $(Ap)_5 (CCp)_3 Cp$, respectively. The CCp content of the fractions appears to decrease with the increase in partition coefficient, whereas the reverse appears to be true for the Ap content of the fractions.

Similarly, solvent systems for the separation of tri and penta nucleotides have been developed. The experiments with trinucleotide separation brought out a very important fact. It has been possible to demonstrate that countercurrent distribution procedures are capable of separating the oligonucleotides having the same base ratios and same chain length, but varying only in nucleotide sequence.

Summary and Conclusions:

In a study of the contribution of labyrinth function to motion sickness, it was observed that normal subjects had markedly increased rates of glucose utilization as well as enzyme changes and alteration of acid-base balance. Labyrinth deficient subjects did not show these responses.

Dietary trans fatty acids were found to be incorporated into rat depot fat and membranes, and were shown to alter membrane functions. The results of the investigation did not indicate an adverse effect of trans fatty acids upon the mammalian organism, but rather suggest a possible functional role for these compounds.

Studies of liver enzyme inactivation of bacterial endotoxins have shown that the lipid moiety of the molecule is essential for toxicity of the endotoxin. Poisoning of the liver by three different hepatotoxins gives varying degrees of damage to the fatty acid oxidation system but not to succinoxidase. When fatty acid oxidation is inhibited, the animal is far more sensitive to endotoxin. It has been shown that varying growth conditions of bacteria alter the quantity but not the toxicity of endotoxin obtained from the bacteria.

Three mutants of E. coli have been isolated relating to carbohydrate oxidation: isocitric dehydrogenase, phosphoenolpyruvate carboxylase, and a third which is characterized by the inability to ferment arabinose and a 30-fold increase of TPNH oxidase.

Control mechanisms of Kreb's cycle enzyme synthesis and activity have been studied. Glucose as a carbon source reduces the synthesis of Kreb's cycle enzymes and increases phosphoenolpyruvate carboxylase.

The difference between certain avirulent and virulent strains of Shigella flexneri is that the virulent strain can penetrate the host cell. The permeability of the avirulent strain to a variety of substrates is considerably less than the virulent one. An investigation into the mechanism of penetration is now in progress.

Using countercurrent distribution procedures, three serine transfer-RNAs from yeast were separated and two of these three were purified. Chemical studies of these two RNAs revealed that Ser. II RNA contains one mole (ApCCp) CCpUp and two moles of ApUp, whereas Ser. III RNA contains two moles of (ApCCp) CCpUp and one mole of ApUp. Several biological similarities among these two RNAs were also observed. Countercurrent distribution procedures for the separation of tri- penta- hexa- and heptanucleotides have been developed. It has been possible to show that this procedure is capable of separating oligonucleotides that vary only in their nucleotide sequences.

Publications:

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PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03
Entomology

RESEARCH AND TECHNOLOGY RESUME	1	2 GOVT ACCESSION	3 AGENCY ACCESSION	REPORT CONTROL SYMBOL
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11 TITLE

(U) Ecology and control of disease vectors and reservoirs (09:21:47:TH)

12 SCIENTIFIC OR TECH AREA	13 START DATE	14 CRIT COMPL DATE	15 FUNDING AGENCY
002600 Biology; 005900 Environ. Biol; 010100 Microbiology	09 54	NA	OTHER | DA

16 PROCURE METHOD	17 CONTRACT GR..NT a DATE / b NUMBER / c TYPE / d AMOUNT	18 RESOURCES EST	a PROFESSIONAL MAN-YEARS	b FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	5	82
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19 GOV'T LAB INSTALLATION ACTIVITY	20 PERFORMING ORGANIZATION
NAME Headquarters	NAME Walter Reed Army Institute of Research
ADDRESS U.S. Army Medical Res & Dev Command Washington, D. C. 20315	ADDRESS Washington, D. C. 20012
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
Public Health	NA

23 KEYWORDS Arboviruses; Chikungunya; Culicidae; dengue; ecology; entomology; insect vectors; malaria (Plasmod. spp.); mosquitoes; taxonomy; tissue culture; vectors and reservoirs

24 (U) Tech Objective - Control of arthropod vectors of dis of mil sig with emphasis on malaria and arbovirus vectors. Efforts are on the incrimination of vectors, anal of host-pathogen relationships, prep of taxonomic monographs on mosquitoes, field studies on vector ecology and the estb of insect cell lines in culture. These studies will permit the ident of med important mosquitoes from strategic areas. Vector efficiency studies should indicate where control measures are best directed. The dev of mosquito
25 cell cultures may be highly significant for in vitro malarial parasite cultivation.

(U) Approach - Mosquito collections are made in the US for arbovirus isol & study of feeding habits. Special overseas collections produce mat for taxonomic studies. Dev of a suitable small animal sys will allow dengue and Chikunguya viruses to be studied in their vectors. The poor in vitro surv of malarial gametocytes has hindered host-pathogen anal & dev of a new chemotherapeutic test system. This may be resolved by finding an aprop holding medium. Attempts are being made to detm which immature stages and tissues of mosquitoes are most amenable to survival in vitro.

(U) Progress (Jul 64 - Jun 65) - Bunyamera group, WEE, EEE & undetm viruses were re-
26 covered from 3 mosquito species in so. Md. and Va. Experimental transmission trials with dengue and Chikunguya viruses are being attempted with Aedes aegypti & Ae. albopictus. The malarial gametocytes exhibits lower in vitro survival than the trophozoite and apparently has different metabolic requirements. Short term survival has been observed with tissues from 3rd and 4th instar mosquito larvae and embryos in culture.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

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35 EST. FUNDS (In thousands)	36
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DD FORM 1 AUG 64 1498 Items 1 thru 24 identical to NASA Form 1121) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 03, Entomology

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

Investigators.
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Description.

This task covers a variety of studies on the ecology and bionomics of arthropods in relation to several groups of pathogenic agents and to a variety of vertebrates involved in infectious disease ecology.

Progress.

1. Ecology of arboviruses in Maryland and Virginia.

Studies on the ecology of mosquito-borne viruses in Assateague - Chincoteague Islands, Va., were carried out during 1964 in continuation of the effort at identifying the arthropod vectors of EEE and the Cache Valley-like virus in that area. In addition, an intensive survey was made in the Delmarva (Delaware, Maryland, Virginia) peninsula to locate the breeding habitat of Culiseta melanura. As a result of this survey the Pocomoke Cypress Swamp, located in Worcester County, Maryland and approximately 20 miles N. W. of Chincoteague, was chosen as an additional study area. Four collecting sites were also chosen between the Pocomoke Cypress Swamp and Chincoteague Bay to check the occurrence of Culiseta melanura in the general area.

Mosquito collections from the Pocomoke Cypress Swamp were obtained by eight battery-operated light traps, ten resting boxes and three bait traps; two with racoons and the other with chicks. All were placed near larval breeding sites. Light traps and resting boxes were operated continuously and the mosquitoes were collected from them three times a week. Over 26,000 mosquitoes representing fifteen species were collected from this habitat. These included both fresh and salt water breeding mosquitoes, but the majority of those collected were Culiseta melanura (89%). Culex salinarius (4%) and Aedes vexans (3%). During the period between June and October five peaks of activity were observed; in mid-June, mid-July, mid-August, early September and early October. The continuous presence of C. melanura and the noticeable overlapping of these peaks suggest that this species is multivoltine at this latitude in Maryland.

Identification of blood-meal sources of 130 engorged Culiseta melanura collected from the swamp was made by the precipitin test. Specimens were tested with ten anti-sera prepared in rabbits. Only 48 specimens gave positive reactions. Results indicated that C. melanura fed on pigs (13), deer (11), racoons (8), reptiles (8), cows (5), dogs (1), horses (1) and man (1). None appeared to have fed on either birds or rodents. Three of these hosts - deer, racoon and reptiles, are present in the swamp while the others are found on farms located within a two-mile radius from this habitat. Feeding of C. melanura on farm animals suggests the possible movements of this species between the swamp and adjacent farms. A few blooded specimens other than C. melanura were also tested. One of five females of Culex salinarius reactioned with anti-reptile serum. Blood meals in single specimens of Aedes canadensis and Aedes vexans could not be identified.

One attempt was made to study the flight range and dispersal pattern of Culiseta melanura. Wild-caught females were collected from the swamp by light traps; from which 1532 females were marked with green fluorescent dust and released soon after at a designated point in the same habitat. Collecting traps were placed in concentric circles at 66 foot intervals from the central point of release. The farthest trap was located 660 feet from the point of release. Ninety-four marked females were recaptured in light traps within a 24 hour period. Since a relatively small number was released and a limited number of traps were employed in this trial, the limits of the flight range could not be established. However, marked female C. melanura were recaptured 15 hours after release in the most distant light trap.

Twelve virus strains were isolated by the Department of Virus Diseases (Project 3A014501B71Q 01 166) from pools of Culiseta melanura. This was the only mosquito species yielding virus (Table I). Eastern equine encephalitis virus was isolated from two pools collected by light traps on September 1. Western equine encephalitis virus was isolated from one pool of mosquitoes collected by the same method on September 30. Viruses were also isolated from nine additional pools of this species collected by light traps and resting boxes during the period between mid-July and mid-September. The identification of these isolates is in progress.

In the woodland habitats of Assateague four racoon-baited traps and twelve battery-operated Chamberlain light traps placed at sites previously selected at random, were used for collecting mosquitoes. These traps were operated on the same schedule followed at the Pocomoke Cypress Swamp study area. In the salt marsh habitats of both islands, mosquitoes were collected once a week by the standardized sweeping method previously developed and tested during 1963. Aedes sollicitans, Ae. taeniorhynchus and Culex salinarius were collected from the marsh habitats. Aedes sollicitans was the dominant species comprising 99% of the total catch. This species completed two generations during the season with one peak of activity

occurring in early August and the other in late September. These data paralleled those of the previous year in frequency and time of occurrence. In the woodland habitat eleven species were found to occur throughout the season, among which Aedes taeniorhynchus, Aedes sollicitans and Culex salinarius were dominant (Table II).

Of the 97 engorged specimens of Aedes sollicitans tested by the precipitin method, 25 gave positive reactions. This species appeared to have fed largely on racoons (7), reptiles (6), man (5), horses (3), birds (2), cows (1) and deer (1). None reacted with dog, pig or rodent antiserum. One specimen of Aedes canadensis gave a positive reaction with bovine antiserum. A single Aedes vexans was negative. One of the five Culex salinarius gave a positive reaction with reptile antiserum.

Over 15,000 mosquitoes collected from both islands were pooled and then processed for virus isolation by the Department of Virus Diseases (Project 3A014501B71Q 01 166). One unidentified virus was isolated from a pool of Anopheles quadrimaculatus. A virus identical to the 1961 and 1963 Cache Valley - Tensaw complex isolates was isolated from each of two Aedes taeniorhynchus pools (Table III).

Light trap collections were made at four monitoring sites between the Pocomoke Swamp and Chincoteague Bay. Weekly collections indicated the presence of sixteen species of mosquitoes. Culiseta melanura were collected from all areas sampled, but in small numbers, not exceeding 5% of the catch of all four sites combined. It is difficult to establish whether adult C. melanura captured in these traps emerged from the immediate vicinity where limited breeding occurred.

2. Viral isolates from sandflies.

Characterization of sandfly virus isolations has continued. The micro haemagglutination-inhibition test confirmed findings of the previously reported neutralization test that two isolates, IP-58 and IP-81 (Annual Progress Report 1 July 1961 - 30 June 1962), from Phlebotomus females collected in Iran were new prototypes and that isolate IS-92 from Sergentomyia females collected in Pakistan was identical with IP-58 (Annual Progress Report 1 July 1963 - 30 June 1964). There are now four serotypes of sandfly fever viruses, including the Naples and Sicilian strains of Sabin, isolated from human sera. The grid HI test was almost completed using mouse immune ascitic fluid prepared against all serotypes and IS-92 (Table IV). Since the high mouse passaged Naples strain (Sabin) did not make a suitable HA antigen, another human isolate from Pakistan was used to prepare the antigen. It was hoped that the infectivity of this strain could be enhanced by the terminal dilution purification method. Work on the HA antigen was terminated when Dengue and Chikungunya virus studies commenced.

Table I

Virus isolations from mosquitoes collected in Pocomoke Cypress Swamp, Maryland during 1964

Species	Total specimens	No. pools tested	No. pools positive
Aedes canadensis	50	2	0
Aedes sollicitans	75	3	0
Aedes vexans	925	22	0
Anopheles bradleyi-crucians	350	14	0
Culex salinarius	850	16	0
Culiseta melanura	23,075	245	12
Total	25,325	302	12

Table II

Mosquito fauna of the woodland habitat of Assateague Island, Virginia as indicated by all collecting methods, 1964

Species	Females collected	Per cent of total catch
Aedes taeniorhynchus	7831	38
Aedes sollicitans	6261	31
Culex salinarius	5311	26
Anopheles quadrimaculatus	488	2
Aedes vexans	168	1
Anopheles bradleyi-crucians	137	1
Aedes cantator	117	1
Culiseta inornata	5	< 1
Aedes canadensis	4	< 1
Mansonia perturbans	2	< 1
Culiseta melanura	1	< 1
Total catch	20,325	100%

Table III

Virus isolations from mosquitoes, collected on Assateague and Chincoteague Islands, Virginia, 1964

Species	Total specimens	No. pools tested	No. pools positive
Aedes cantator	25	1	0
Aedes sollicitans	5100	57	0
Aedes taeniorhynchus	4725	60	2
Aedes vexans	75	3	0
Anopheles bradleyi-crucians	75	3	0
Anopheles quadrimaculatus	400	16	1
Culex salinarius	5075	62	0
Total	15,475	202	3

Table IV

Haemagglutination inhibition[1] with immune mouse ascitic fluid

	Antigen				
Ascitic fluid	Naples	Sicilian	IP-[illegible]	IP-81	IS-92
Naples		0	2	0	2
Sicilian		5	0	0	0
IP-58		0	4	2	5
IP-81		0	2	5	2
IS-92		0	5	2	4

[1] HI titer of ascites is expressed by a number that represents the highest serial twofold dilution of ascites that gave complete inhibition of haemagglutination. 1:10 = 1, 1:20 = 2, 1:40 = 3, 1:80 = 4 and 1:160 = 5.

3. Experimental transmission of Dengue and Chikungunya viruses.

The program has been devoted mainly to finding a suitable host and donor animal for infection of aedine mosquitoes with strains of Dengue and Chikungunya viruses. The membrane feeding technique is also being evaluated as a means of infecting the mosquitoes.

Trials with different routes of inoculation and different dosages did not give detectable, circulating Dengue 2 virus (obtained from Department of Virus Diseases, WRAIR) in adult mice and hamster weanlings. Adult mice were inoculated subcutaneously (SC) with 0.03 ml. and intraperitoneally (IP) with 0.2 ml. of 10^{-2} dilution of Dengue 2 virus and hamster weanlings SC with 0.03 ml. and 0.1 ml. of a 10^{-1} dilution. Attempts to induce viremia in mice with Freund's adjuvant, hyaluronidase, gastric mucin, E. coli endotoxin, adrenccortical hormone and cold stress also gave negative results. One-half ml. of Freund's adjuvant (complete) was injected IP and 2 hours later, 0.03 ml. of 10^{-3} dilutions of Dengue 2 virus were inoculated SC into two series of mice. These animals were bled daily for 14 days for detection of circulating virus. A 20% suckling mouse brain (smb) suspension was mixed with hyaluronidase (final concentration 20 units/ml.) and 0.03 ml. of the mixture inoculated SC. Mice were bled daily for two weeks. A 20% smb suspension was mixed with an equal amount of 5% gastric mucin. A series of mice were inoculated SC with 0.05 ml., 0.15 ml. and 0.2 ml. of the mixture and mice were bled daily for 14 days. Mice were injected IP with 0.2 ml. endotoxin (200 microgram), inoculated 2 hours later SC with 0.03 ml. of a 10^{-1} dilution of virus and mice bled daily for 14 days. Five mg. of adrenocortical hormone was administered to adult mice SC 2 hours prior to SC inoculation of two series of mice with 0.03 ml. of 10^{-1} and 10^{-3} virus dilutions and mice bled from days 4 through 10. Mice were inoculated SC with 0.03 ml. of 10^{-1} and 10^{-3} virus dilutions, half were kept in a biological oxygen demand box at 4°C. to simulate cold stress and half of the inoculated mice were kept at ambient temperature. Both series of mice were bled from days 4 through 10. Aedes aegypti mosquitoes were fed a chicken blood-virus (Log_{10} LD_{50} 6.2 per 0.03 ml. of blood) by the membrane feeding technique. Three mosquitoes were titrated within an hour after their infectious blood meal (Day 0) and the quantity of virus ingested was 50, 100 and 159 LD_{50} respectively (Log_{10} LD_{50} of 1.7, 2.0 and 2.2 per 0.03 ml. of blood). Titrations on days 1-14, 21, 28 and 35 were negative. Attempts at mosquito transmission to baby mice were unsuccessful.

Inoculation of adult mice by the intramuscular, IP and SC routes with 0.05 ml. and 0.1 ml. of 10^{-5} and 10^{-6} dilutions of the African strain (Ross) of Chikungunya virus did not produce detectable viremia. Both the African and Asian (3113-62) strains were then tested in weanling hamsters. Seven transmissions of virus A. aegypti from hamsters to suckling mice have occurred; two with the African strain and five with the Asian strain. The transmissions will be confirmed by neutralization or serological tests. A. aegypti were infected on days 1

and 2 after inoculation of the hamsters with the African strain and were infective 14 and 16 days after their blood meal. Mosquitoes infected on days 21, 23, 25 and 26 with the Asian strain were infective 6, 14-16 and 20 days after their infectious blood meal. It appears that the mosquitoes are able to pick up circulating virus from the hamsters sooner with the African strain (although one hamster circulated Log_{10} LD_{50} 5.0 per 0.02 ml. of blood on day 4). Incubation periods were similar in the two strains. The African strain is a "high mouse passaged" strain and the Asian is "low mouse passaged." Transmission attempts with a new strain of A. aegypti are projected.

4. Factors affecting in vitro survival of malarial gametocytes.

Viability of malarial gametocytes can be only demonstrated by their ability to produce the sporogonous cycle when ingested by an appropriate mosquito. The membrane feeding technique (Annual Progress Report 1 July 1963 - 30 June 1964) has made it possible to manipulate the blood forms of plasmodia in vitro with subsequent mosquito ingestion. During the year, a number of studies have utilized the Plasmodium gallinaceum - Aedes aegypti - chick blood system.

Initially, heparin sodium solution (100 U.S.P. units/ml. blood) was used as an anticoagulant for membrane feeding experiments of infected blood. There was a consistent reduction in gametocyte infectivity with this material, particularly after refrigeration of infected blood for periods of two hours or longer. The alternate use of defibrination of blood with glass beads produces no change in gametocyte infectivity.

For chemotherapy experiments it is often necessary to have a large pool of infected blood for mosquito infection. Usually, an infected chick can provide 10 ml., an amount sufficient for only two feeders. Recent work indicates that blood may be pooled from several infected birds and that the resultant mixture has an infectivity equivalent to the average of each separate contributor.

Infected blood may be diluted with normal blood with no reduction in mosquito infectivity. In a recent experiment (Table V) there was an indication that mosquito infectivity is actually enhanced by dilution. This may be a consequence of dilution of immune or other inhibitory substances in the sera of infected hosts which affect gametocyte infectivity.

Defibrination of blood does not alter its acceptability to mosquitoes in the membrane feeding apparatus and digestion apparently occurs normally in the mid-gut of the mosquito host.

5. Immunization of chicks against Plasmodium gallinaceum malaria.

Attempts at artificial immunization of chicks against fowl malaria by various investigators have shown varying results. In an attempt to

Table V

Susceptibility of *Aedes aegypti* to diluted *Plasmodium gallinaceum*

Percentage Infected blood	Percentage Normal blood	No. of mosquitoes	Mean no. oocysts ± S.E.
100.00	0	25	14.2 ± 4.3
50.00	50.00	25	19.6 ± 9.1
25.00	75.00	24	21.3 ± 4.3
12.50	87.50	25	27.5 ± 6.5
6.25	93.75	25	34.8 ± 6.7

repeat some of the earlier studies, the following antigens have been tried; heavily parasitized blood inactivated by storage at 20°C., for 60-120 days; the same suspended in an equal volume of Freund's incomplete adjuvant by sonification; and ground, infected mosquitoes in which the sporozoites had been inactivated by treatment with 13,000 rep of gamma radiation. Inoculation of 100-150 gram white leghorn chicks was intramuscular in one milliliter amounts (equivalent to six ground mosquitoes in the case of the last antigen). Depending on the particular experiment, two or three inoculations were administered at intervals of three to seven days prior to sporozoite challenge. Criteria for recognition of immunity were length of prepatent period, number of days to peak parasitemia, number of days to 50% mortality and number of deaths. To date, these antigens have not afforded demonstrable protection. Future studies will utilize only inactivated sporozoites with greater quantities used and longer periods of time between administration of antigen and challenge.

6. Effects of chemosterilants on malarial parasites.

As stated in the 1964 report, certain alkylating agents can interrupt the development of Plasmodium in the mosquito and at the same time induce mosquito sterility without abnormal mortality. This year's efforts have been directed toward the development of an efficient, reliable method for screening the activities of these compounds. Plasmodium gallinaceum and Aedes aegypti were used in tests of apholate, tepa, metepa and hempa. After a blood meal on an infective chick, mosquitoes were transferred to jars or tubes coated inside with a measured deposit of chemosterilant. They were later transferred to holding cages for observation. At day six, per cent mortality, per cent egg hatch and malarial oocyst count were determined.

The pint jar with screw cap was found to be an impractical treatment chamber. Because of the irregular shape of the jars, it is difficult to apply a uniform deposit of chemosterilant on their inner surfaces. As a result, mosquitoes may adhere to the jar where concentrations of the chemosterilant are present. The fumigant effect is also a source of error because the jars are completely closed and the chemosterilants differ in volatility. Other methods of mosquito exposure have been examined. The treatment and holding tubes of the World Health Organization insecticide resistance test kit for adult mosquitoes are screened at one end and have a plastic slide for mosquito transfer at the other. This design overcomes the main difficulties experienced with the pint jars. These tubes were designed for use with insecticide-impregnated paper linings. The use of filter and typewriter bond paper impregnated with chemosterilants gave erratic results. To overcome this problem, standard 40 mm. glass tubing cut to 125 mm. lengths were utilized as liners. These are easily treated and can be removed for cleaning prior to re-use. The WHO test kit tubes with glass liners have been used in all recent experiments.

The residual activity of most chemosterilant deposits diminishes with time. It has been found best to treat the tubes on the afternoon preceding the day of the test. One hour after infection, the mosquitoes are placed in the test chambers for a period of three hours. Twenty-five or thirty mosquitoes are needed for each tube. Following exposure, they are transferred to observation cages for six days.

The results of the experiment of 2 April 1965 are presented in Table VI. Complete sterilization of the mosquito and marked suppression of plasmodial development were given by tepa and metepa; however, metepa was moderately toxic to the mosquito. Less striking results were given by apholate and hempa. In general, concentrations of these four particular chemicals that completely prevent oocyst development and egg hatch are also toxic to some degree.

7. Mosquitoes of Southeast Asia.

This project has been initiated as a cooperative endeavor between the U. S. National Museum (a bureau of the Smithsonian Institution) and the Department of the Army to make a definitive study of the mosquito species present in Southeast Asia. Final publications will include illustrations and descriptions of adult and immature stages, taxonomic keys, an ecological and biological profile of each species, and, where pertinent, specific detailed information on the role of each species in the transmission of human and/or animal diseases. The project will be devoted to the assembly of available information on the mosquito fauna of the region, study and evaluation of material presently housed in the U. S. National Museum, British Museum of Natural History, and other scientific repositories. To supplement existing collections, field collections are presently being made in selected localities to provide ecological and biological data as well as additional study material. Results of these studies will be initially published in sections, as completed, in order that the information will be available for use by the military. The ultimate goal is the publication of a monographic work including all available systematic, ecological, biological, and zoogeographical information available for the area under consideration.

The material that has been collected by Major John E. Scanlon and other personnel at the SEATO Medical Laboratory has been brought to the U. S. National Museum where it, together with the regular Museum collection and substantial numbers of as yet unstudied Thailand specimens, is being prepared and studied. Additional specimens will be either borrowed from other museums or studied at those museums. During this period, personnel of the U. S. Component, SEATO Medical Research Laboratory, U. S. Army Medical Research Unit, Malaysia and the Walter Reed Army Institute of Research will make additional field collections in Southeast Asia, rear field collected specimens, and gather additional biological and ecological data concerning selected species. The illustrations for this project will be made primarily by the Zoological Artist Group at the

Table VI

Effects of chemosterilants on Aedes aegypti and Plasmodium gallinaceum

Chemosterilant	Concentration (mg ft^2)	No. of mosquitoes	Mean no. oocysts ± S.E.	Six day mortality	% of eggs hatching
Control	0	25	65.2 ± 23.1	0/25	99
Tepa	5	24	12.8 ± 7.4	1/25	0
Metepa	10	21	0.3 ± 0.3	3/24	0
Apholate	10	25	51.6 ± 19.8	1/26	-
Apholate	20	21	39.2 ± 18.0	0/21	67
Hempa	50	25	51.6 ± 20.4	0/25	26

406th Medical General Laboratory under the supervision of Lt Colonel H. L. Keegan but it is expected that a portion of this work will have to be performed at the U. S. National Museum in Washington. Specimens, data and illustrations ultimately will be assembled and the project completed at the U. S. National Museum, where the major portions of the project will be under the direction of Dr. Alan Stone, USDA, Dr. Ralph A. Bram, Smithsonian Institution, and Major John Scanlon, WRAIR. Selected specialist consultants will be required to assist in the studies on various groups of mosquitoes and, whenever feasible, military entomologists will participate in this project as technical assistants either in the laboratory or field portions of the work.

A considerable amount of time and effort during the first year of the project was devoted to construction, organization, recruitment of qualified personnel, and liaison with members of the scientific community. Laboratory facilities and office spaces have been constructed at the Lamont Street annex of the Smithsonian Institution. Arrangements have been completed for personnel to fill all staff positions and training of preparators and museum aids has been successfully accomplished. In cooperation with the Military Entomology Information Service, and the various Federal libraries, over 10,000 pages of reprints and abstracts devoted to the mosquito systematics of the area have been accumulated.

Studies of the mosquito fauna of Khao Yai National Park, Thailand, have been completed and the manuscript is now being prepared. Additional studies have been initiated which deal with the following genera: Anopheles, Culex and Toxorhynchites.

Tentative keys to the adult and immature stages of the mosquitoes of Viet Nam have been prepared and made available to interested military personnel.

Summary and conclusions.

1. As in 1963, no evidence was obtained for the dissemination of EEE virus on Assateague and Chincoteague Islands during 1964. Two isolations of Cache Valley-like virus were obtained from Aedes taeniorhynchus and one unidentified virus from Anopheles quadrimaculatus. Observations on population dynamics of salt-marsh and woodland mosquitoes were continued during 1964. Two strains of EEE virus and one of WEE were isolated from Culiseta melanura from the swamp. These findings constitute the first record of WEE isolation from Maryland and the first record of isolation of EEE from mosquitoes in the same state. These results suggest that Culiseta melanura might be the principal enzootic vector of EEE and WEE viruses in that area. It may be possible that during epidemics, exchange of arboviruses between these habitats and Chincoteague could occur since C. melanura is prevalent in the mainland between these areas. Studies on the population dynamics of Culiseta melanura indicated

the continual presence of the species in the fresh water swamp and its possible movements between this habitat and adjacent farms.

2. Isolates IP-58 and IP-81, recovered from sandflies of the genus Phlebotomus in Iran were confirmed as new prototypes by the micro haemagglutination-inhibition test. This test also verified that isolate IS-92 from Sergentomyia sandflies from Iran was identical with IP-58.

3. Dengue-2 virus, adapted to weanling mice by 31 passages, does not circulate in adult mice or hamster weanlings. Replication of the virus in aedine mosquitoes has not been demonstrated. Seven unconfirmed transmissions of two strains of Chikungunya virus by Aedes aegypti from weanling hamsters to suckling mice have occurred.

4. The membrane feeding technique for mosquitoes is being used to investigate factors affecting in vitro survival of malarial gametocytes. Sodium heparin solution reduces gametocyte infectivity to mosquitoes, but defibrination produces normal development. Pooling of blood from several infected chicks yields an infectivity equivalent to the average of each separate contributor. Infected blood may be diluted with normal blood to as little as 6.25% without loss of infectivity to mosquitoes.

5. Attempts to repeat some of the earlier studies on immunization of chicks against fowl malaria have not resulted in demonstrable protection. Radiation-killed sporozoites will be used as the antigen in future attempts.

6. A procedure for using the World Health Organization insecticide resistance test kit for adult mosquitoes in studies on the effects of insect chemosterilants on malarial parasites has been developed. Glass linings were found to be superior to paper linings in the treatment tubes of the kit. Chemosterilant activity and insecticidal activity of the chemical under test are measured simultaneously with the effect on the malarial parasite. Tests of apholate, hempa, tepa and metepa show that the latter two produce the greatest reduction in malarial oocyst count and per cent egg hatch with the least mosquito mortality. The most effective concentrations for tepa and metepa were 5 and 10 mg/ft^2, respectively.

7. A definitive study of the mosquitoes of Southeast Asia has been initiated in cooperation with the U. S. National Museum. The U. S. Component of the SEATO Medical Research Laboratory, the U. S. Army Medical Research Unit in Malaysia and the Zoological Artist Group of the 406th Medical General Laboratory will also participate. The ultimate goal is the publication of a monographic work including all available systematic, ecological, biological and zoogeographical information on the mosquitoes of Southeast Asia. Construction, organization and recruitment for the project are nearly completed. A large number of specimens has been brought together, and over 10,000 pages of reprints and abstracts have been accumulated. Studies of the mosquito fauna of Khao Yai National Park,

Thailand, have been completed and a manuscript is being prepared. Tentative keys to the adult and immature stages of the mosquitoes of Viet Nam have been prepared and made available to interested military personnel.

Publications.

Gould, D. J., Byrne, R. J. and Hayes, D. E.: Experimental infection of horses with Japanese encephalitis virus by mosquito bite. Amer. J. Trop. Med. Hyg. 13:742-746, 1964.

Rutledge, L. C., Ward, R. A. and Gould, D. J.: Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. Mosquito News 24:407-419, 1964.

PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04
Immunology

RESEARCH AND TECHNOLOGY RESUME

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21 TECHNOLOGY UTILIZATION: NA

22 COORDINATION: NA

23 KEYWORDS: Allergy; enzymes; immunology; antigen; antibody; hypersensitivity

24 (U) Tech Objective - Work under this work unit involves the study of the basic mechanisms of the immediate type allergies from the viewpoint of the enzyme systems involved. This looks to the ultimate control of such allergies by a specific inhibition of these enzymes. The development of methods for the quantitative determination of antigen and antibody concentrations in absolute weight units. These methods will be applicable to the assay of natural mixtures of antigens and antibodies. The application of quantitative cell counting techniques to the characterization of blood group antigens and antibodies is another important aspect of the work under this work unit.

25 (U) Approach - The peptidase nature of certain of the components of the complement system are being studied. Phosphonate inhibitors are being synthesized and tested in vitro for selective action against various enzymes. The various factors affecting the use of anti-sera in quantitative gel-precipitin tests are being investigated. The distribution of blood group antibody between fluid phase and human erythrocyte is being investigated under various conditions of temperature and concentration of cells and antibody.

26 (U) Progress (Jul 64 - Jun 65) - Sheep erythrocytes containing one of the third components of hemolytic complement have been shown to be capable of enzymatically splitting glycyl L tyrosine. Contrary to previous reports the binding affinities of antibodies for the human B-anti-B system are definitely heterogeneous.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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

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DD FORM 1498 (Items 1 to 36 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

Investigators.

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Description.

The purpose of this task is to study the phenomena involved in the agglutination reaction of the human blood group system, the enzymatic mechanisms of allergic reactions, and the quantitation of gel precipitin reactions.

Progress.

1. Phosphonate Ester Inhibition of Rabbit C'1a

a. The same p nitrophenyl ethyl phosphonate inhibitors used to characterize guinea pig activated first component were tested against rabbit C'1a. EAC'1a4 in which the C'1a was from rabbit and C'4 from guinea pig serum were employed for this purpose.

b. The relative changes of inhibitory activity with the changes in the structure of the phosphonate parallelled those found with guinea pig C'1a, except in one instance. With guinea pig C'1a, the benzyl phosphonate was found to be a more active inhibitor than the phenylphosphonate, whereas with the rabbit C'1a, the reverse was true.

2. Phosphonate Inhibition of Antigen Induced Histamine Release from Sensitized Rat Peritoneal Mast Cells

Studies have begun of the inhibition by phosphonate esters of the antigen induced release of histamine from sensitized rat peritoneal mast cells. The inhibition found is similar in broad outlines to that found with sensitized guinea pig lung, but there are distinct differences which are under present investigation.

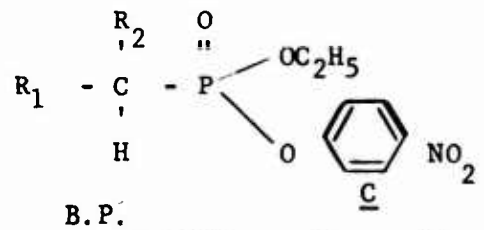
3. The Synthesis of Phosphonate Esters

The preparation of new phosphonate inhibitors is continued. Seventeen hitherto unknown and unreported compounds are recorded in Table I

4. Association of Peptidase and Complement Activity

The results of inhibition studies of immune hemolysis and immune adherence reported in the preceding Progress Report, suggested that one of the C'3 components involved in the formation of the 37°C stable intermediate was a peptidase. Following this suggestion, it was shown by paper chromatography that cells prepared from EAC'142 and whole guinea pig serum at 0° hydrolyzed aromatic amino acid containing dipeptides, among them glycyl tyrosine. The reaction gave a bell shaped pH curve with an optimum at pH 7.6. Hydrolysis followed zero order kinetics. The velocity of the reaction was linear with cell concentration and its variation with substrate concentration followed simple Michaelis-Menten kinetics. The Km was 1.1×10^{-3}M. The peptidase activity in isotonic tris buffer pH 7.6 could be reduced by adding 0.01 MEDTA or by incubating the cells at 37° for two hours followed by washing before adding substrate. EAC'142 did not hydrolyze glycyl tyrosine. In general, there appeared to be increasing hydrolysis with increasing immune adherence activity.

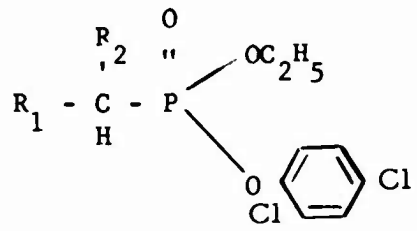
TABLE 1



Empirical Formula	R_1	R_2	B.P. (C°)*	n_D^{25}	C T	C F	H T	H F	H T	H F	P T	P F
$C_{19}H_{18}NO_5P$	-Naphthyl	H	184	1.6091	61.5	60.9	4.9	5.3	3.8	3.1	8.3	7.9
$C_{19}H_{18}NO_5P$	-Naphthyl	H	212	1.6068	61.5	61.4	4.9	4.9	3.8	3.1	8.3	8.6
$C_{15}H_{24}NO_6P$	Pentyl	Methoxy	138	1.5117	52.2	52.6	7.0	7.0	4.1	4.1	9.0	8.4
$C_{17}H_{28}NO_6P$	Heptyl	Methoxy	153	1.5051	54.7	54.9	7.6	7.6	3.8	3.5	8.3	8.0
$C_{17}H_{20}NO_6P$	Phenethyl	Methoxy	153	1.5486	57.0	56.9	5.9	5.9	3.7	3.8	8.2	8.2
$C_{15}H_{22}NO_7P$	Propyl	Carbethoxy	110	1.5012	50.1	50.4	6.2	6.3	3.9	4.1	8.6	8.4
$C_{17}H_{20}NO_7P$	Phenyl	Carbethoxy	80	1.5054	53.5	54.1	5.3	5.4	3.7	3.5	8.1	4.0

*Molecular still

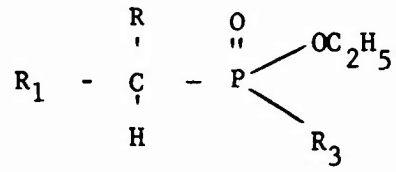
TABLE 1



Empirical Formula	R_1	R_2	B.P. (°C)	n_D^{25}	C		H		Cl		P
					T	F	T	F	T	F	
$C_{14}H_{19}Cl_2O_5P$	Propyl	Acetoxy	128(0.1)	1.5021	45.5	45.3	5.2	5.3	19.1	19.3	8.4	8.6
$C_{19}H_{21}Cl_2O_5P$	Phenethyl	Acetoxy	189*	1.5350	52.9	52.8	4.9	5.0	16.4	16.6	7.2	6.7

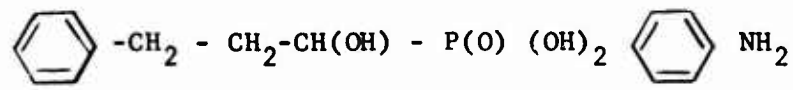
*Molecular still

TABLE 1



Empirical Formula	R_1	R_2	R_3	n_D^{25}	B.P. °C	C T	C F	H T	H F	N T	N F	P T	P F
$C_6H_{12}O_3P$	Cyano	H	Ethoxy	1.4316	82-84(0.05)	-	-	-	-	-	-	-	-
$C_{11}H_{15}O_5P$	Phenyl	Acetoxy	Hydroxy	-	189*	51.2	51.4	5.9	6.1	-	-	12.0	12.1
$C_{11}H_{23}O_5P$	Propyl	Carbethoxy	Ethoxy	1.4325	99-100 (0.15)	-	-	-	-	-	-	-	-
$C_9H_{18}ClO_4P$	Propyl	Carbethoxy	Cl	1.4469	82-84(0.05)	-	-	-	-	-	-	-	-
$C_{14}H_{21}O_5P$	Phenyl	Carbethoxy	Ethoxy	1.4918	134(0.02)	56.0	55.8	7.1	7.0	-	-	10.3	10.2
$C_{14}H_{22}NO_5P$	Phenyl	Carbethoxy Amino	Ethoxy	1.5047	155(0.1)	53.3	53.7	7.0	6.9	-	-	9.8	9.9

*Molecular still



$C_{15}H_{20}NO_4P$					171-173	58.2	58.1	6.5	6.2	4.5	4.8	10.1	10.2

5. Release of Kallikrein during Anaphylaxis in the Isolated Perfused Guinea Pig Lung

The perfusate from isolated sensitized guinea pig lung, following addition of antigen to the perfusion fluid, is capable of forming kinin from dog plasma pseudoglobulin (Brocklehurst, W. E. and Lahiri, S. C. J. Physiol. 160: 15P, 1962). We have confirmed this observation and have further shown that the active principal corresponds in its properties to plasma kallikrein. The isolated lungs of guinea pigs passively or actively sensitized to bovine serum albumin were perfused free of blood, and antigen was added to the perfusion cannula. The perfusate during the first 5 minutes after antigen did not contain kinin as assayed by the estrus rat uterus, but was capable of releasing kinin from plasma substrate heated to 61° for 30 minutes, dialyzed to remove spontaneously formed kinin, and acidified to inactivate kininase. The perfusate caused increased capillary permeability in guinea pig skin, also inhibitable by soybean trypsin inhibitor.

6. Permeability globulin system

The course of activation by glass of permeability activity in intact, undiluted human plasma and its subsequent inhibition was studied. A peak of permeability activity occurs within five to ten minutes after placing the plasma in glass and disappears within thirty minutes to two hours. The fact that soybean trypsin inhibitor, 50 μg/ml, completely inhibits the activity and that heparin 500 units/ml inhibits it 21% indicates that 55% of its activity is caused by kallikrein and 45%, by Pf/dil. In some experiments, although not in all, a second peak of permeability activity occurs after the plasma has stood in glass for four hours. The reasons for the inconstant occurrence of this second peak of activity is not understood. Since soybean trypsin inhibitor completely inhibits this peak and heparin 500 units/ml inhibits it 97%, Pf/dil accounts for practically all of its activity.

The disappearance of the two peaks is caused by the presence of inhibitors whose activity can be diluted out.

The behavior of kallikrein and Pf/dil on Sephadex Gel Columns was investigated. On Sephadex G200, kallikrein appears in the trough between 19S and 7S protein and is not retarded by Sephadex G100. This behavior agrees with the sedimentation constant of around 11S found in previous work from this laboratory. On Sephadex G200, Pf/dil appears in the trough between 7S protein and is not retarded by Sephadex G100. This behavior agrees with the sedimentation constant of around 5.2 found in previous work from this laboratory. On Sephadex G200, Pf/dil appears in the trough between 7S protein and albumin and spreads into the albumin range, as expected from its sedimentation constant of 5.2S. If partially purified Pf/dil prepared from DEAE chromatography of Cohn Fraction I + III 1, 2, 3 is placed on Sephadex G100, it will be retarded and separated from most of the contaminating protein which comes through in the void volume. Recycling of this Pf/dil yields a product which is 1750 fold purified over plasma in terms of bluing activity.

7. Rabbit passive cutaneous anaphylaxis

The injection of either bovine gamma globulin or dinitrophenyl bovine gamma globulin with complete Freund's adjuvant in the foot pads of adult rabbits results in the formation in 15%-30% of the animals of an antibody which produces passive cutaneous anaphylaxis (PCA) in normal rabbits. The antibody appears on the 7th to 8th day following immunization and by the 14th to 18th day is no longer detectable. PCA reactions are obtained only after the antibody has been in the skin of the rabbit for 48 hours, and peak reactions require a latent period of 72 hours before the intravenous injection of antigen and pontamine sky blue. The PCA activity migrates in electrophoresis faster than IgG globulin and sediments more slowly than IgM globulin in sucrose density gradient. PCA activity is destroyed by freezing and thawing the antiserum or heating at 56° for two hours, or treatment with 2 mercaptoethanol. The resemblance to rat PCA antibody and human reagin is clear.

8. Histamine release from rabbit thrombocytes by an antigen-antibody reaction

The investigation of the multicomponent nature of the histamine releasing system in rabbit plasma was continued with emphasis on the portion(s) of the system specifically adsorbed on to antigen-antibody precipitates, the so-called precipitate factor.

a. Antigen-antibody precipitates allowed to adsorb precipitate factor and then incubated with di-isopropylfluorophosphate, DFP, show partial inhibition of precipitate factor activity.

b. Incubation of plasma treated precipitates with EDTA (ethylenediamine tetra acetic acid) partially deprived them of precipitate factor activity.

c. Precipitates treated with plasma heated to 56° for one-half hour were unable to restore histamine releasing activity to a supernatant plasma.

9. Quantitative precipitin reactions in Oudin tubes: the effect of treatment of antisera on the optical density

In a continuation of studies of the quantitative gel precipitin method described previously, the manner in which various treatments of the antiserum affect the optical density of the leading edge of the precipitate band has been investigated. Optical density was found to decrease with length of time of storage of antisera at 4°C up to approximately one week after which time there was no further decrease; the O.D. decrease was correlated with the hemolytic complement activity of the antisera. Antiserum heated at 56°C for 0 to 20 minutes showed first a marked decrease in O.D. then an increase to approximately the O.D. value originally observed. Antiserum absorbed with an unrelated immune precipitate gave O.D. values less than those observed with the unadsorbed antiserum. When the adsorbed antiserum was heated at 56°C for 0 to 20 minutes only an increase in O.D. was observed. The results suggest the presence in freshly drawn antiserum of both a heat labile enhancer and a heat labile inhibitor of the gel precipitin reaction which are possibly related to complement.

10. Studies of the reactivities of iso-agglutinins with A_1 and B red cells

a. The studies of the reactivities of anti-A and anti-B sera with A_1 and B red cells were completed. Previous findings that the relative activities of A_1 and B antigens depend on the antiserum employed were confirmed.

The relative agglutinating strengths of four B cells observed with seventeen anti-B sera are shown in Tables IIa and IIb, respectively. Immunization of two group O and four group B donors with hog A and human A_1 and A_2 saliva substances did not alter the pre-immune patterns of reactivity, indicating that the diverse behaviour of the anti-A sera is not related to the specificity of the isoagglutinin.

b. It was concluded that there exists a heterogeniety of A_1 and B red cell antigens of such complexity that it is impossible to evaluate or assign any genetic interpretation to the results of quantitative studies of these antigens.

c. Immunization of a group O donor with group B substance caused a rise in cross-reactive 7S anti-A antibodies along with a change in the reactive pattern suggesting that the variable behaviour of isoagglutinins may be related to the proportions of 7S and 19S isoantibodies in the serum. Studies of the whole serum and serum fractions eluted from a DEAE-cellulose column showed that the relative strengths of the three standard A_1 cells were 100%, 75% and 50% respectively with the whole serum; 100%, 100% and 50% with the fraction containing 7S antibodies; and 100%, 65% and 50% with the fraction containing 19S anti-A antibodies. Although this suggests that a mixture of the 7S and 19S agglutinins could result in the reactive pattern seen with the whole serum, additional information is needed.

11. Thermodynamic studies of the A-anti-B system

In the early studies of the binding affinities of naturally occurring anti-B isoagglutinins, the hemagglutinating activity of supernatants from the absorptions of antisera with varying B cell concentrations at 37°, 25° and 4°C were measured at room temperature. Such variations in results of the 4°C absorptions were observed that interpretation was impossible. These studies have been extended using a wider range of absorbing cell concentrations (3000-48,000 cells/mm^3) and with supernatants tested at 4°C. When, as suggested by Klotz, the reciprocal of the antibody fixed per cell was plotted against the reciprocal of the antibody free, these data gave straight lines with slopes that increased in the same order for all

Table 2a

Pattern of Behavior	Antiserum	Relative Reactivity of A_1 Red Cell Antigens		
		A_1(F.C.)	A_1(J.T.)	A_1(L.B.)
I	4 commercial sera 2 natural group O sera	100%	75%	50%
II	4 commercial sera	100%	100%	50%
III	2 natural group O sera 4 natural group B sera 2 immune group O sera 4 immune group O sera	100%	100%	70%
IV	1 immune group O serum	100%	100%	40%
V	1 immune group O serum	100%	90%	75%

Table 2b

Pattern of Behavior	Antiserum	Relative Reactivity of B Red Cell Antigens			
		B(C.P.)	B(J.B.)	B(D.H.)	B(F.M.)
I	4 commercial sera	100%	100%	60%	60%
II	2 commercial sera 3 natural group A sera 2 immune group A sera	100%	80%	50%	50%
III	1 natural group O serum 3 immune group A sera	100%	80%	60%	40%
IV	1 natural group O serum	100%	80%	40%	60%
V	1 natural group A_x serum	100%	65%	50%	50%

antisera; it could therefore be concluded that the great variation in slopes of the 4°C absorption curves observed in the initial experiments reflects the inhomogeneity of these natural isoagglutinins.

b. Using the method of plotting suggested by Scatchard, in which the ratio of the antibody fixed per cell to the antibody free is plotted against the antibody fixed per cell, the data obtained by assaying supernatants at 4°C gave essentially the same anomalous results as previously observed with the room temperature measurements. The slope and configuration of the curves depended on the concentration of cells used in absorption. These findings also strongly suggest that the binding affinities of anti-B antibodies from apparently unstimulated individuals are not homogeneous, in contradiction to the conclusion of the Wurmsers..

c. Inhomogeneity of the naturally anti-B antibody was confirmed by absorbing a group O serum with high and low cell concentrations (48,000 and 3000/mm^3) and then eluting the bound antibody at 56°C. Subsequent absorption of the two eluates and the whole serum with three different B cell concentrations revealed striking differences in the behavior of these antibody populations. When the reciprocal of the antibody fixed per cell was plotted against the reciprocal of the antibody free, the data for the whole serum gave three distinct curves of variable slopes; the data for the eluate from the high cell concentration gave three curves with similar slopes; and all data from the absorption of the eluate from the low cell concentration fell on a single straight line indicating an apparent homogeneity of this antibody population. This is supported by the results of the Scatchard plot where the data for this eluate also gave a single straight line.

d. Absorptions of antisera from three group O, three group A_1, two A_2 and an A_x individual were carried out at 37 and 25°C and the hemagglutinagins activities of supernatants tested at 4°C. When the data were treated in the plot suggested by Klotz, the ratio of the slopes of the 37 and 25°C curves differed for each antiserum. This is not consistent with the Wurmsers' findings of a constant slope ratio for sera from individuals of a given ABO genotype.

12. Quantitative studies of the Rh antigens of a family postulated to possess an Rh inhibitor gene.

The studies of the Rh antigens of members of a family postulated to possess an inhibitor gene acting on the biosynthetic pathway of Rh antigens, conducted in collaboration with Dr. Philip Levine of the Ortho Research Foundation, were completed. The red cells of the propositus exhibited no Rh activity (Rh_{null} phenotype or ---/---) but she transmitted the CDe complex to her child whose genotype is CDe/cde. Quantitative assays of the C, D, c, e and f, c, e antigens of the family have shown that only four out of 16 members of her kindred (two paternal uncles, one maternal uncle and a sibling) possess Rh antigens comparable in strength to the antigens of standard control cells. The Rh antigens of the other twelve family members were considerably weaker than those of standard donors of the same genotypes. The observation that four maternal siblings sharing only two CDe and two cde genes manifested three grades of C, D and c activities when only two grades are possible provided definitive evidence that the genetic mechanism which has caused the Rh_{null} condition of the homozygous propositus is also operating to a lesser extent in the heterozygous state; a mode of gene action, heretofore, not described for human blood group systems.

13. Quantitative studies of the S and s antigens of the Rh_{null} family

a. Dr. Paul Schmidt of the NIH Clinical Center has found the blood of the Caucasian Rh_{null} propositus described above lacks both S and s antigens, as well as Rh antigens, suggesting that the genic interaction causing her Rh_{null} condition may also inhibit expression of Ss genes on a chromosome segregating independently from the Rh chromosomes. A collaborative study has been undertaken on the S and s antigens of the bloods of the relatives of the propositus which have been preserved in liquid nitrogen in our laboratory. Using cells from normal donors, the conditions for the assay of anti-S and anti-s sera have been investigated. Thus far, only the anti-S assay has proved satisfactory in that reproducible differences between the agglutinating strengths of homozygous and heterozygous cells are demonstrable.

14. Quantitative studies of the LW antigens of human erythrocytes

a. The log-probit assay method of Wilkie and Becker has been applied to the measurement of the LW activities of human erythrocytes using a human anti-serum, a guinea pig anti-rhesus red cells serum and a guinea pig anti-baboon red cell serum. Assay curves for the two guinea pig anti-sera were parallel but the curves for the human anti-serum were found to be considerably flatter.

b. Assay of the LW antigens of 20-D-positive cells from a variety of Rh genotypes with the human and guinea pig anti-LW sera and with anti-D serum showed that LW activity parallels the D reactivity of human red cells. This indicates that LW antibodies are reacting with some portion of the structure of D determinants. These findings are not compatible with the contention of Levine that "LW determinants are not phenotypically related to Rh antigens but are the products of independent LW genes."

15. Measurement of the osmotic fragility of red cells by a recording densitometer

In collaboration with Major C. E. Shields of the Department of Hematology, Walter Reed Army Institute of Research, a test tube holder and a dialysis-frame specimen cuvette were designed for the Spinco Analytrol which permits the continuous recording of the change in density of a suspension of red cells exposed to continuously decreasing salt concentrations. The designed attachments cost less than $200 as compared to $1600 for the commercial Fragilograph. The modified recording densitometer was found to give reproducible measurements of the osmotic fragility of red cells. The instrument has been applied to a study of the osmotic fragility of normal and abnormal human and animal red cells.

Summary and Conclusions.

1. The inhibition of rabbit C'1a by the same phosphonate inhibitors used to inactivate guinea pig C'1a was studied. Except for minor differences the pattern of inhibition given by the two species was the same.

2. The phosphonate inhibitors have been shown to inhibit antigen induced histamine release from sensitized rat peritoneal mast cells.

3. The synthesis of phosphonate esters is continued.

4. Peptidase activity associated with complement activity has been studied.

5. The kallikrein released during anaphylaxis in the isolated perfused guinea pig lung has been shown to correspond in its properties to plasma kallikrein.

6. The injection of either bovine gamma globulin or dinitrophenyl bovine gamma globulin with complete Freund's adjuvant into the foot pads of normal adult rabbits results in the formation of an antibody which produces passive cutaneous anaphylaxis in the rabbit. This antibody corresponds in its properties to the rat PCA antibody and human reagin.

7. A peak of permeability activity occurs within five to ten minutes after placing intact, undiluted plasma in glass and which disappears within two hours. Kallikrein is retarded on G200 Sephadex, but not on G100, whereas, Pf/dil is retarded on G100.

8. The portion of the histamine releasing system from rabbit plasma found on the precipitate is heat labile, and inactivated by EDTA and DFP.

9. There exists a heterogeneity of A_1 and B red cell antigens of such complexity that it is impossible at this time to evaluate or assign any genetic interpretation to the results of quantitative studies of these antigens.

10. Thermodynamic studies of the binding of human anti-B natural isohemagglutinins with B red cells reveals that such antisera contain a population of antibodies with a distinct heterogeneity of binding affinities.

11. Quantitative studies of the Rh antigens of a family postulated to possess an Rh inhibitor gene has suggested a mode of gene action heretofore not described for human blood group systems.

12. Quantitative studies of the S and s antigens of an Rh_{null} family suggests that genic interaction causing this Rh condition may also inhibit expression of the Ss genes on another chromosome.

13. Quantitative studies on the LW antigens of human erythrocytes indicate that LW antibodies are reacting with some portion of the structure of the Rh (D) determinant.

14. The Spinco Analytrol was modified to permit continuous measurements of the osmotic fragility of human red cells.

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RESEARCH AND TECHNOLOGY RESUME

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C. IN-HOUSE	c TYPE NA	PRIOR FY 65	2	69
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19 GOVT LAB INSTALLATION ACTIVITY
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21 TECHNOLOGY UTILIZATION	22 COORDINATION
NA	NA

23 KEYWORDS: Chikungunya; Rift Valley fever; vaccines; immunology

24 (U) Tech Objective - Chikungunya is an arthropod-borne viral disease that is indigenous to Africa and Asia. Outbreaks of epidemic proportions have been reported from Africa, Southeast Asia and India. It is a seriously debilitating, though rarely fatal disease of man. The highest mortality occurs in persons under 12 years of age. This investigation is primarily concerned with the production of a vaccine offering broad spectrum protection for man.

25 (U) Approach - Despite its widespread geographical distribution, Chikungunya virus is characterized by several closely related strains. Cognizance of these close antigenic relationships enhanced the feasibility of preparing a formalin-killed vaccine with one well-characterized virus strain.

26 (U) Progress (Jul 64 - Jun 65) - This vaccine has been assayed in rhesus monkeys for its immunogenic potency and broad spectrum capacity against the homologous and several heterologous strains of the virus. Results have shown that the vaccine fulfills both requirements to an excellent degree. The vaccinated monkeys were completely protected against challenge with African, Thai, and Indian strains as well as the homologous African strain; whereas the non-vaccinated control group became infected. Three additional lots of vaccine are currently being evaluated for potency and stability. Five consecutive lots of Rift Valley fever vaccine have been produced to meet current requirements.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27 COMMUNICATIONS SECURITY	28	29 OSD CODE	30 BUDGET CODE
a COMSEC OR COMSEC RELATED X b NOT RELATED		BR	1

31 MISSION OBJECTIVE	32 PARTICIPATION
NA	NA

33 REQUESTING AGENCY	34 SPECIAL EQUIPMENT

35 EST FUNDS (In thousands) FY	36

DD FORM 1498 AUG 64 — Items 1 to 26 identical to NASA Form 1122. REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 016, Immunization studies of exotic diseases

Investigators.
Principal: R. Randall, DVM
Associate: V. R. Harrison, MS

Description.

This task is concerned with the development, production and evaluation of formalin-killed vaccines against the agents of Chikungunya and Rift Valley fever (RVF).

Progress.

During the current report period work has been directed primarily toward the evaluation of two lots of vaccine prepared in African green monkey kidney tissue infected with the 168 strain of Chikungunya virus and inactivated with 1:1000 formalin (USP 37%). Despite its wide geographical distribution throughout Africa, Southeast Asia and India, this virus is characterized by several closely serologically related strains. Cognizance of these close antigenic relationships enhanced the feasibility of producing a vaccine against one well characterized strain (168) which would confer protection against other members of the Chikungunya complex. A preliminary assay of mouse sera following 2 doses of vaccine, 0.2 ml at 7-day intervals utilizing the serum bead neutralization test (SBNT), indicated that broad protection was afforded against the homologous as well as 3 heterologous strains from Africa (E-103), Thailand (BAH-306), and India (63-266), of the Chikungunya virus.

On the basis of these preliminary observations, one group of 8 rhesus monkeys was given three 1 ml doses of this vaccine subcutaneously. A second group of monkeys was held as non-vaccinated controls. Thirty days after the third dose of vaccine the monkeys were divided into 4 groups and challenged subcutaneously with approximately 10^6 SMICLD$_{50}$, intraperitoneally with either the homologous or 3 heterologous strains of African, Thai and Indian origin.

The animals were temperatured and bled for 5 consecutive days to detect any febrile response and for the presence of circulating virus. At intervals of 15, 30, and 65 days following challenge all of the monkeys were bled for serologic evaluation.

Following challenge the vaccinated animals remained afebrile with no detectable viremia, whereas, the non-vaccinated control group, with

a single exception* exhibited a temperature rise and a patent viremia of 2 to 5 days' duration (See Table I). Results of the serologic evaluation were characterized by a sharp elevation in CF and HI titers, and substantial increases in the zones of inhibition by the SBNT for the non-vaccinated group, whereas, no significant elevation in CF or HI response, or increase in the size of the zones of inhibition (SBNT) were observed in the vaccinated group (See Table II).

Results of this assay in rhesus monkeys indicate that excellent protection against the homologous as well as 3 heterologous strains of Chikungunya virus is conferred by a formalin-killed vaccine prepared with a well characterized strain of the Chikungunya complex.

In order to determine the stability of the Chikungunya vaccines after storage and lyophilization, two lots of fluid and lyophilized vaccine are currently being assayed for potency in mice. One lot was held for 2½ years in the fluid state at 4C while the other lot was held for five months, aliquots of both lots were then lyophilized.

The presence of neutralizing antibody to Chikungunya in the sera of wild-caught non-human primates as reported by Kokernot and McIntosh in Africa, has stimulated interest in screening recent non-human primate imports by means of the SBNT at this installation. A total of 68 sera from non-human primates representing 5 species, i.e., chimpanzee, gorilla, orang, baboon and rhesus monkey, have been screened for the presence of neutralizing antibody to Chikungunya and Rift Valley fever. All sera tested were negative for RVF . Forty-four percent of the sera tested indicated the presence of neutralizing antibody to Chikungunya. Twenty-two and 29% were positive for CF and HI antibody, respectively (See Table III).

Five additional lots of RVF vaccine have been prepared for human use. As described previously four lots were lyophilized and one lot maintained in the fluid state. Excellent potency values were obtained for these lots.

Summary and Conclusions.

The need for a vaccine against Chikungunya is well documented by the fact that outbreaks of epidemic proportions have been reported from Africa, Thailand and India. Although the disease is rarely fatal in adults it is a seriously debilitating one. Two lots of vaccine against this virus have been prepared and assayed in mice and rhesus monkeys. Results indicate that the vaccines are high in potency and

* The fact that one of two non-vaccinated monkeys challenged with Chik-168 (African strain) failed to become infected is not unusual. In a communication with Dr. Hankins, AMU, Ft. Detrick, Md., he informed us that only 43% of their rhesus monkeys challenged with this strain of Chikungunya became infected.

confer broad-spectrum protection against the homologous strain as well as three heterologous strains of African, Thai and Indian origin. A survey of 68 non-human primate imports revealed a 44% incidence of reactors to Chikungunya by means of the SBNT . The detection of CF and HI antibody in many of these sera suggests the possibility of recent infection. Thus it would be considered advisable for importers to observe strict quarantine precautions for these animals, particularly in areas of high mosquito density.

In order to meet current requirements five additional lots of RVF vaccine have been prepared. All lots gave excellent potency values.

TABLE I

Viremia Studies in Rhesus Monkeys Following Challenge with Selected Strains of Chikungunya Virus

MK	Vaccine	Challenge	Plasma inoc. in African Green Monkey Kidney Tissue Culture ($TCID_{50}$/0.1 ml)				
#	Status	Virus	1	2	3	4	5 (day)
603	Yes	Chik-168[a]	0	0	0	0	0
604	Yes	Chik-168[a]	0	0	0	0	0
647	No	Chik-168[a]	0	0	0	0	0
650	No	Chik-168[a]	0	0	2.5	1.5	0
605	Yes	Chik-E103[b]	0	0	0	0	0
606	Yes	Chik-E103[b]	0	0	0	0	0
651	No	Chik-E103[b]	3.3	4.8	4.5	2.8	0
652	No	Chik-E103[b]	3.5	>5.5	4.8	3.5	0
615	Yes	BAH-306[c]	0	0	0	0	0
645	Yes	BAH-306[c]	0	0	0	0	0
653	No	BAH-306[c]	0	3.8	4.5	1.2	0
656	No	BAH-306[c]	2.3	4.5	4.5	2.8	1.5
646	Yes	63-266[d]	0	0	0	0	0
648	Yes	63-266[d]	0	0	0	0	0
659	No	63-266[d]	0	3.8	3.8	0	0

a = African strain
b = African strain
c = Thailand strain
d = Indian strain

TABLE II

Serological Response of Rhesus Monkeys Following Challenge with Selected Strains of Chikungunya Virus

MK	Vaccine	Challenge	Days Post Challenge								
			15			30			65		
#	Status	Virus	CF	HI	BNT*	CF	HI	BNT	CF	HI	BNT
603	Yes	Chik-168	8	20	14	4	20	14	4	10	16
604	Yes	Chik-168	8	20	16	8	10	14	4	10	14
647	No	Chik-168	0	0	0	0	0	0	0	0	0
650	No	Chik-168	128	160	20+	512	640	20+	64	160	20+
605	Yes	Chik E103	4	20	15	0	20	15	0	10	15
606	Yes	Chik E103	4	20	17	0	10	14	0	10	15
651	No	Chik E103	512	1280	18	512	5120	16	512	640	20+
652	No	Chik E103	512	1280	20+	512	2560	20+	128	1280	20
615	Yes	BAH-306	0	10	16	0	10	14	0	10	14
645	Yes	BAH-306	4	10	12	4	10	13	0	10	12
656	No	BAH-306	128	160	20	128	160	19	64	160	19
653	No	BAH-306	>512	640	18	>512	640	19	128	640	19
646	Yes	C-266	0	0	11	0	0	12	0	0	12
648	Yes	C-266	4	20	13	4	10	12	0	10	12
659	No	C-266	64	320	15	256	160	15	64	80	20

*BNT = Bead Neutralization Tests (Zone of plague inhibition measured in mm.)

TABLE III

A Serological Survey of Non-human Primates for the Presence of Antibody to Chikungunya

No. of Animals	Source	SBNT* Pos	SBNT* Neg	CF Pos	CF Neg	HI Pos	HI Neg
24	Yerkes Lab	10	14	1	23	1	23
39	WRAIR	17	22	12	27	17	22
5	AMU Ft.Detrick	3	2	2	3	2	3

* Serum Bead Neutralization Test (Zone of plaque inhibition measured in mm.)

Publications.

Raymond Randall, L. N. Binn and V. R. Harrison. Immunization against Rift Valley Fever virus: Studies on the immunogenicity of lyophilized formalin-inactivated vaccine, Journal of Immunology, 93:293-299, 1964.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6434	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY RPT / WRK	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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PRIOR FY 65	6	177
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19. GOV'T LAB/INSTALLATION/ACTIVITY
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INVESTIGATORS PRINCIPAL ASSOCIATE: Einheber, A., Ph.D.
Rosen, H., Ph.D., Carter, Capt. D.
TEL: 202-576-3636 TYPE: DA

21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Antibiotics; bacteria; biochemistry; burns; germfree life; metabolism; pathology; radiation; shock; surgery; uremia; wound healing

24. (U) Tech Objective - By use of germfree (GF) biotechnology, conduct multidisciplinary comparative studies on GF, defined-flora ex-germfree, and conventional (ized) animals to isolate the bacterial from the non-bacterial (host) factors and delineate their beneficial or detrimental effects on the body economy in health or after injury, shock and disease states induced by various agents and stressors and to learn to manipulate the indigenous and environmental microorganisms or their effects to the best advantage of the host.

(U) Approach - Studies are continuing on: antibacterial vs. non-antibacterial (toxic) actions of antibiotics; alterations of GI tract by microbial flora; immunological response of lymphatic system after antigens and liver damage; hormonal effects (adrenal steroids, alloxan diabetes); amino acid and protein metabolism and enzyme induction; skin grafting and wound healing; shock induced by limb or bowel ischemia and burns; anuric states (biochemistry and survival with uremia); derivation, maintenance and study of GF primates (monkey).

(U) Progress (Jul 64 - Jun 65) - We have found that microbial status of the host significantly influences morphology, GI physiology, protein metabolism, and response to chlortetracycline, antigens, bowel ischemia, uremia and wound healing. Fatal abacterial peritonitis occurs in GF rats from a leaking suture line of the cecum. Hyperoncotic 10% Low Molecular Weight Dextran is not an effective blood substitute for the monkey after hemorrhagic shock. Irrespective of microbial status, saline therapy improves survival time after bowel or limb ischemia or burns.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 017, Responses of germfree animals

Investigators.
Principal: Albert Einheber, Ph.D.
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Description.

Study phases are in progress or are completed on:

1) The germfree animal, its technology, production, rearing, maintenance, and nutrition; to evaluate and exploit its potential in biologic research, and its use in experimental procedures which create conditions that are of interest to and have practical importance for military medicine.

2) Comparative analyses of the constitution (anatomy and biochemistry) and function (behavior, physiology, metabolism) of intact germfree, defined-flora, and conventional(ized) animals.

3) Comparative analyses of the responses (constitution and/or function) of germfree, defined-flora, and conventional(ized) animals after challenge with physical, chemical or viable noxae, or combinations thereof; to isolate the bacterial from the non-bacterial (host) factors and delineate their beneficial or detrimental effects on the body economy in health or after injury, shock and disease states; and to learn to manipulate the indigenous and environmental microorganisms or their effects to the greatest advantage of the host.

Progress.

Germfree Animal Production and Utilization:

During the past year, 1,364 germfree animals were used for investigative purposes. The source and species of animals are as follows:

Obtained from Charles River Breeding Laboratories:

Rats - 360
Mice 900

Born and reared germfree or delivered by Caesarean in this Department:

Fischer Rats - 92
BALB/c Mice - 11
African Green Monkey - 1

Germfree Technology

Anesthesia - A system which provides anesthesia for surgical and other procedures on germfree rodents within a germfree surgical isolator has been devised and successfully used in this Department. The pre-selected air-halothane mixture is sterilized by passage through spun glass filters. A small lucite chamber and a nose cone allow induction and maintenance of the halothane anesthesia within the isolator. The system has provided a safe, reproducible anesthesia from which recovery is brief and from which there is no explosion hazard. A manuscript is in press in the Journal of Applied Physiology for publication in May 1965 on this system.

Fabrication of an Isolator Designed for Metabolic Studies - Until now, careful experiments of a metabolic nature have not been carried out in isolators because of the impossibility of making periodic urine collections without repeatedly preparing sterile locks for urine removal, with the attendant hazard of contamination of the isolator. Moreover, certain labile compounds deteriorate in urine if it is not kept cold or otherwise preserved. In addition, there is a requirement peculiar to germfree research, viz., that no bacterial action be allowed to change any component of the urine of the conventional animals in their isolators because the germfree urine is necessarily sterile and remains so. To allow bacterial action in the conventional urine would make impossible any interpretations of *in vivo* differences between germfree and conventional animals.

In order to make possible metabolic experiments, we have designed and constructed an isolator which allows urine to be funneled off into narrow plastic sleeves which can be sealed off as consecutive samples of urine are taken. Four such sleeves attached to one isolator service four animals in metabolic cages; the latter effectively separate the excreted fecal pellets from the urine. The plastic sleeves hang freely from the floor of th isolator; the urine enters one end of the sleeve which is sealed to and opens into the floor of the isolator. The other end of the sleeve, where the urine collects, is sealed off. The sleeves are kept in small refrigerators so that the urine is kept cold at all times; if necessary, the sleeves can be cooled in dry ice.

Prototype "Biological-Fallout" Shelter for Monkeys - To minimize the risk of airborne infection of our ex-germfree monkey (see elsewhere in report) with pathogens, we have devised and fabricated a monkey cage with a fiberglass filter-material roof, walls of glass covered inside with wire mesh, and a pre-filtered air supply delivered under positive pressure into the shelter.

Needed improvements in design have become obvious with experience and, if accomplished, this unit will become generally useful for housing monkeys.

Dry Fiberglass Air-Outlet Filters for Steel Isolators - Liquid air-exhaust traps have been in use on 16 of our stainless steel isolators. These were found to have questionable efficiency. To provide better protection against contamination during the initial sterilization of the steel isolators, these liquid outlet traps have been replaced with dry fiberglass filters. This has standardized the type of air-inlet and air-outlet filters used in this laboratory and reduces maintenance cost.

Disposable Trays for Diet Sterilization - The light metal trays in which L-356 diet is steam-sterilized for use in isolators have been of the reusable type. These have been replaced by light aluminum disposable trays. In the past, considerable time was spent in washing and scraping the baked diet from the reusable food trays. This changeover allows greater economy of space in the autoclave lock during removal of the new trays because they can be easily bent and crumpled, greater certainty of adequate sterilization of food that is entered into isolators because only new trays are used, and reduces the overall time spent in preparation of diet, and the number of autoclaved entries into isolators.

Modification of Containers Housing Rodents in Isolators - The perforated stainless steel lids of the steel containers which have served to house animals within isolators have not permitted viewing of the animals. These have now been replaced with clear plastic perforated lids. This enables investigators and technicians to observe the experimental animals without the need for entering isolators, thus minimizing the attendant hazard of glove "breaks" and contamination. This also aids the technician in servicing the units. Together with this change, plastic dividers that compartmentalize the steel containers are now also available; these provide individual housing of animals when required.

Microbiology

Routine Bacteriological Monitoring of Animal Isolators - An improvement in the monitoring of germfree animal isolators was instituted by use of sterile brain-heart infusion broth (BHI) in 5 ml aliquots for the purpose of moistening Calgiswabs before sampling the diet, bedding and stool. The broth permits a more effective sampling of these specimens and tends to increase the period of survival of the microorganisms between the time of collection and culturing. Thus, the effectiveness of monitoring is increased.

A further improvement in monitoring has been achieved by the utilization of several additional selective media to enhance the rapidity and accuracy with which microorganisms can be identified. These media include SS (Salmonella-Shigella) agar, Tergitol-7 agar, Buffered Azide Glucose Glycerol broth (BAGG) combined with agar for plating, bismuth sulfite agar, lactobacillus selection broth combined with agar for plating, tomato juice agar, staphylococcus medium No. 110, and PPLO (pleuropneumonia-like organisms) enrichment broth combined with agar for plating.

In conjunction with this approach to monitoring, the specific isolation of Bacteroides species, which consist of fastidious, gram-negative, non-sporeforming anaerobic bacilli, was investigated. A review of the literature indicated that several media have been tried. To determine which might be the best for our purpose, we obtained two Bacteroides species from the NIH, where they were isolated from "pathogen-free" mice. The media tested included ordinary blood agar (5% sheep's blood) as well as a medium developed by Goldberg, Barnes and Charles (J. Bact. 87:737, 1964) which consisted of 10g peptone, 5g NaCl, 3g beef extract, 5g yeast extract, 0.4g cysteine hydrochloride, 6g agar per liter of distilled water. This medium was modified by using 15g agar per liter for plating. In addition, another batch of the medium was plated as a modification which included neomycin (20 ml/L of a 1:10 diluted stock solution consisting of 0.5g neomycin sulfate/50 ml distilled water), to inhibit facultatively-anaerobic gram-negative bacilli commonly found in intestinal microflora, and crystal violet (0.005%/L). Finally, the medium was also tested when modified by the addition of 40% ox bile since Beerens and Castel (Ann. Inst. Pasteur 99:454, 1960) reported the stimulating effect this biologic displayed with Bacteroides cultivation in vitro. The anaerobic cultivation of the two Bacteroides species revealed that ordinary sheep blood agar plates were far superior to the other media tested, based on a visual observation of the degree of growth. The modification consisting of neomycin and crystal violet failed, however, to support the growth of the cultures tested.

The mechanics of anaerobic cultivation have been greatly facilitated by the recent installation of a Torbal jar evacuation and gas charging (with CO_2 and H_2) apparatus in this Department.

Improvement in Quantitative Determination of Microflora in Cecal Contents and Fecal Pellets - The major problem in quantitative studies with this type of biological material is the accurate determination of the number of bacterial cells per gram of dry weight cecal contents or fecal pellet. It is desirable to use small accurately weighed quantities of these biologics to reduce the extent of the dilution series needed for countable plates (i.e., to save time, glassware and dilution fluid as well as reduce chances of contamination which increase with excessive handling of these materials), and to have quantities which can be easily processed. It is also desirable to have a reliable standard upon which to compare data from several animals and, especially, different experiments.

Thus, with these goals in mind, a solution was suggested which has so far proved feasible in its practical application. For cecal contents, an ordinary platinum milk loop is flamed, allowed to cool briefly and is then impaled in a small block of polyvinyl sponge (a material that has practically negligible weight for its volume). These are weighed on a Mettler analytical balance. The loop is removed and sterilized via Bunsen burner. The sterile loop is then used to scoop up cecal content, re-impaled in the sponge and weighed. The cecal content is suspended in 1.0 ml of dilution fluid. From this point, the cecal content can be carried through a dilution series before plating. Present studies reveal that usually a 10^3 to 10^4 final dilution is sufficient for achieving countable plates. The remainder of the cecal contents can be placed in a tared weighing dish

without any necessity for aseptic technique. This material is weighed and then dried and reweighed. In this manner, the water content of the cecal material can be ascertained and, hence, its dry weight. After the dilutions are taken into account, the colony counts can be converted to cells per gram dry weight of cecal content.

The above procedure can also be used for fecal pellets, with a platinum (or other metal) wire used to impale this dry, formed material instead of a loop. The fecal pellet, is first cut in half with a sterile scalpel blade. One-half is weighed and processed for cultivation while the other half is used for dry weight determination. (This is based upon the assumption that the fecal pellet is rather uniform in consistency with as much water content in one-half as in the other.)

Both the loop and the wire can be more easily manipulated with a hemostat than in their respective holders during the weighings. The possibility of aerial contamination still exists, but if all manipulations are performed rapidly and within a confined area of the laboratory, this problem is reduced.

Effect of Tyrosine Upon the Intestinal Microflora of Open-Room Conventional Rats Receiving Large Tyrosine Loads - Unpublished work done by this Department has shown that vitamin C-deficient germfree guinea pigs, when given loads of tyrosine by mouth, excrete p-hydroxyphenylpyruvic acid in ever-increasing amounts as the tyrosine administrations continue. This is to be expected in view of the known function of ascorbic acid in the intermediary metabolism of tyrosine. However, conventional, vitamin C-deficient guinea pigs reacted to this same administration of tyrosine quite differently; at first, the urinary p-hydroxyphenylpyruvic acid appeared, but lessened with each dose of tyrosine and finally almost disappeared. We postulated that some component of the gastrointestinal flora might be adapting to the tyrosine load in the conventional guinea pigs, thus deaminating or decarboxylating the tyrosine, or merely sequestering it; thus the load might never reach the liver and would not be available for conversion to intermediates with their subsequent urinary excretion. Instead, the bacterial products of tyrosine would be mainly excreted in the feces.

In order to test this hypothesis, we decided to repeat the forced-feeding of rats with tyrosine and to study the effect of this feeding on a specific microorganism and on cecal size.

A review of the literature has revealed that Streptococcus faecalis rapidly decarboxylates free tyrosine (Gale, E.F., Adv. in Enzymol. 6:1, 1946) For this reason, an attempt was made to determine if the concentration of this microorganism would increase in the intestinal microfloral population of conventional rats receiving an excessive amount of tyrosine.

Three-month-old male conventional rats were used in the pilot experiment. They weighed, on the average, 300g. They were given either 0.75mg per gram body weight per day of tyrosine by intubation, or an equivalent volume of saline in two daily doses. These doses were given every other day. The animals were allowed L-356 diet and water ad libitum.

A specific synthetic medium for assaying Streptococcus faecalis (Kihara, Klatt and Snell, J. Biol. Chem. 197:801, 1952) consisting of 18 amino acids, 7 vitamins, and several inorganic salts, was pre-tested before the experiment. The common microorganisms found in the normal rat intestine, in addition to S. faecalis, were tested. Unfortunately, all microorganisms grew as well as the S. faecalis. Thus, it was decided that in addition to the synthetic medium, blood agar and blood agar plus neomycin (15 μg/ml) would be employed.

After the rats had received three intubations, two tyrosine-treated animals and one control were sacrificed and their cecal contents diluted serially and plated via the overlay agar technique (Adams, Methods in Med. Res. 2:1, 1950) on the aforementioned media. The cultures were incubated anaerobically in Torbal jars at 37°C. for 24 hours. Colony counts were made. The following results were obtained:

Media	Tyrosine-treated*		Control*
	I	II	
Blood agar	$.7 \times 10^6$	0	3.1×10^7
Blood agar + Neo.	3.3×10^6	1.6×10^6	3.2×10^7
"Tyrosine" medium	2.3×10^6	1.5×10^6	3.6×10^7

* S. faecalis expressed as cells/g dry wt. of cecal contents.

The data indicate that there is no dramatic difference between the tyrosine-treated rats and the control. A less than ten-fold difference cannot be considered significant. Actually, the results appear to indicate that there are fewer microorganisms in the tyrosine-treated rats than in the control, perhaps suggesting some degree of inhibition. Further investigations would be needed to confirm the data.

On the other hand, the tyrosine administration appeared to definitely increase cecal size (up to 20% compared to saline controls). We are investigating this phenomenon.

Conventionalization: Role of Aerobic and Anaerobic Flora on the GI Tract and Cecal Reduction

Work continues on the development of a suitable gnotobiote, i.e., an animal with a defined-flora that satisfies the criteria of an open-laboratory conventional animal. The availability of such an animal is essential for evaluating the specific role of various microorganisms on host nutrition and metabolism. The difficulty in developing a suitable gnotobiote derives from the fact that there is no constancy in the flora of open-laboratory conventional animals and to the lack of specific criteria for judging the degree to which an ex-germfree animal has been rendered "conventional." One obvious criterion is the reduction of cecal size from germfree proportions (6-15% of the total body weight) to that of conventional animal size (normally less than 2% of the total body weight). Other criteria which might be used are: elevation of the serum gamma globulin level, and histological appearance of the GI tract and lymphatic tissue. We have been attempting to assemble a bacterial flora which will satisfy these and other criteria that we may determine to be useful.

Role of Aerobic Bacteria in Cecal Reduction - Our method for conventionalizing germfree animals has been and still is the introduction of a suspension of cecal content containing a mixed microbial flora into predesignated isolators. This cecal content is obtained from "pathogen-free" animals of the WRAMC colony. Although we have no assurance that the components of the flora of this cecal suspension do not change, either qualitatively or quantitatively from one time to the next, it does consistently reduce the germfree cecum to the size of that of conventional animals. We have, therefore, attempted to isolate individual bacteria from this suspension and to administer them, either singly or combined, for purposes of determining which of these are responsible for reducing the cecum to normal size. To date, none of the aerobes we have tried has caused any reduction in the size of the cecum. The aerobic bacteria we have used are: Aerobacter sp., Bacillus cereus, Bacillus circulans, Bacillus subtilis, Escherichia coli, Proteus sp., Staphylococcus albus, and Streptococcus faecalis. In addition, the yeast Rhodotorula as a mono-contaminant and in combination with an anaerobe, Bacteroides sp., has failed to reduce the size of the cecum.

Role of Anaerobic Bacteria in Cecal Reduction - It has been reported (Skelly, B.J. et al, PSEBM 110:455, 1962) that Clostridium difficile (A.T.C.C. 90556), a gram-positive anaerobe, causes reduction in size of the cecum of gnotobiotic mice. This same report also mentions that two strains of Bacteroides, when used together, also cause a reduction in the size of the cecum. Bacteroides are gram-negative, obligate anaerobic bacilli. We attempted to corroborate the above finding using Cl. difficile. A suspension of Cl. difficile (A.T.C.C. 90556) was obtained and placed in a cooked meat broth. Cultures were taken of these bacteria to assure ourselves of their viability.

Conventionalization by Open-Room Exposure vs. Effects of Cl. difficile in Mice - Male and female mice monocontaminated with B. circulans were divided into three groups: Group I was allowed to remain monocontaminated; Group II was exposed to a suspension of Cl. difficile that was spread throughout their food, water, bedding and cage within their isolator; and Group III was conventionalized by being removed from their isolators and placed in our open conventional rodent room. All mice were sacrificed and examined 2 and 4 days post-contamination. All mice were killed by carbon dioxide inhalation and weighed. (The word "sac" or "cecal sac" will be used to refer to the cecum devoid of its contents; the word "cecum" will refer to the cecal sac plus its contents.) The cecum was carefully extirpated and weighed. The cecal contents were removed under aseptic conditions. A portion of this was inoculated in freshly poured blood agar plates and incubated in an anaerobic phosphorus chamber for 24-48 hours at 37°C. Routine qualitative analysis of the cecal content was made to detect the presence of aerobic bacteria. Blood agar plates, MacConkey's and Sabouraud's agar plates, thioglycolate broth and trypticase soy broth were incubated at 37°C., 55°C. and at room temperature for these tests. The empty sac was weighed and then fixed in 10% buffered formalin for histologic examination.

The following results were obtained:

Group	Purposeful Contamination*	Wt. of Cecum Plus Contents as % of Body Weight 2 da	4 da	Wt. of Cecal Sac Only as % of Body Weight 2 da	4 da
I	None	6.4 (10)	6.1 (10)	0.9 (10)	0.7 (10)
II	Cl. difficile	3.6 (10)	3.2 (6)	1.2 (10)	1.4 (6)
III	Open-Room Exposure	2.2 (10)	1.6 (10)	0.7 (10)	0.8 (10)

Number of animals used in parentheses.
*All mice were monocontaminated with B. circulans at the beginning of experiment.

The ceca of mice that were placed in the open rodent room are significantly smaller than those of mice exposed to Cl. difficile. The cecum of the Cl. difficile-contaminated mice was significantly smaller than that of mice allowed to remain monocontaminated with B. circulans. However, all mice that received Cl. difficile had severe diarrhea. Eight of the 20 mice used in this group did not survive the full course of the experiment. The survivors were much weakened at the time of sacrifice. The weight of the cecal sac (also expressed as a percent of the body weight) was

significantly larger in the Cl. difficile group than in either of the other two groups. We ascribed this to inflammation and edema of the wall of the cecum which was confirmed on histologic examination.

Cecal Histology of Cl. difficile vs. Open-Room-Exposed Mice - The ceca of mice monocontaminated with B. circulans gave no morphological evidence of being different from that of a completely germfree animal. Addition of Cl. difficile caused a definite acute enteritis with damage greater than that from contamination by open-room exposure. Mucosal damage, acute inflammation and edema were more marked at 2 and 4 days after contamination with the organism than after a similar period following uncontrolled exposure to the mixed microbial flora of the conventional environment.

Conventionalization by Open-Room Exposure vs. Effects of Cl. difficile in Rats - Male and female rats were divided into the four following groups: Group I consisted of germfree rats; Group II consisted of rats that had been bicontaminated with B. cereus and B. subtilis; Group III consisted of bicontaminated rats that also received Cl. difficile (A.T.C.C. 90556); and Group IV consisted of germfree and bicontaminated rats that were placed in our open conventional rodent room for a period of 1 to 28 days. The rats of Group III were exposed to fresh suspensions of Cl. difficile at weekly intervals after the first exposure. The killing and culturing procedures were the same as those used above in the mouse study. All of the rats in Group I were killed 14 days after the beginning of the experiment. There was no significant difference in the weight of the cecum or the cecal sac between the rats of Group I and Group II. The rats of Group II were removed and killed at 14, 21 and 28 days, as were the rats of Group III. The following results were obtained;

Grp.	Purposeful Contamination	Days Post-Contamination 0	1	3	7	10	14	21	28
		Wt. of Cecum Plus Content (% Body Weight)							
I	None[a]						11.7 (8)		
II	None[b]						11.3 (6)	11.8 (8)	12.4 (11)
III	Cl. difficile[c]						4.5 (6)	7.8 (8)	8.9 (13)
IV	Open-Room Exposure[d]	8.1 (6)	5.9 (6)	2.1 (6)	1.9 (7)	1.7 (7)	1.6 (16)	1.8 (8)	1.2 (8)
		Wt. of Cecal Sac Only (% Body Weight)							
I	None[a]						1.1 (8)		
II	None[b]						1.0 (6)	0.9 (8)	0.8 (12)
III	Cl. difficile[c]						1.1 (6)	0.8 (8)	0.7 (13)
IV	Open-Room Exposure[d]	0.8 (6)	0.9 (6)	0.7 (6)	0.5 (6)	0.4 (7)	0.3 (16)	0.3 (8)	0.3 (8)

See next page for footnotes to this table.

No. of rats used in parentheses.
[a]Germfree at start of experiment and at sacrifice; [b]Bicontaminated at start of experiment and at sacrifice; [c]Bicontaminated at start of experiment; [d]Bicontaminated or germfree at start of experiment - combined as there was no significant difference in cecal size.

Contamination with Cl. difficile reduces the cecum to a size that is significantly smaller than that found in either the germfree (Group I) or bicontaminated (Group II) rats throughout the entire span of the experiment. However, there appears to be a general trend upward in the size of the cecum as time progresses for the Cl. difficile-contaminated rats (Group III). This is not the case with rats that were exposed to the mixed microbial flora of the open conventional room (Group IV). The reduction in size of the cecum is apparently permanent in the latter case. The cecum of open-room conventionalized rats is significantly smaller than that of any other group of rats including those exposed to Cl. difficile. In addition, the cecal sac of the open-room exposed rat is significantly smaller than that of all other groups from 14 days post-contamination until the experiment was terminated.

Cecal Histology of Cl. difficile vs. Open-Room-Exposed Rats - The open-room conventionalized rat cecum displays at 1 day, an increased cellularity of the lamina propria, glandular narrowing and a slight degree of acute inflammation. By 3 days, the cecal mucosal pattern had progressed markedly towards that typical of the conventional animal. By 7 days, the submucosal edema and inflammation had largely subsided and the mucosa was almost fully conventional in appearance. By 10 days, the mucosa was fully conventional. This pattern persisted throughout with only an occasional appearance of focal scarring of the submucosa. The Cl. difficile-contaminated rat cecum by 2 weeks was generally conventional in appearance, but somewhat thicker. By 3 weeks, the mucosa had thinned out and assumed some of the characteristics associated with germfree ceca, such as wider glands. By 4 weeks, the cecum had further progressed to a more germfree-like appearance.

In summary, although Cl. difficile, as it was studied by us, did reduce the size of the cecum of the gnotobiotic mouse and rat, as reported by Skelly et al., it is obvious that it does not replicate the pattern of cecal changes obtained after contamination with a mixed-microbial flora. The marked diarrhea which occurs early after exposure to Cl. difficile appears largely to account for this reduction in cecal weight. While our routine conventionalization procedure produces a reduction in the weight of the cecal sac per se, Cl. difficile, by contrast, produces an edema and an increase in cecal sac weight. Moreover, the reduction in the size of the cecum of the rat after 2 weeks of contamination with Cl. difficile was less than that obtaining after open-room exposure, and unlike the latter, began to wane progressively over the next 2 weeks. The mechanism underlying the progressive re-enlargement of the cecum in the presence of ample Cl. difficile is not known.

A similar observation was reported by Wiseman and Gordon (Nature, 205: 572, 1965). These investigators have demonstrated bioactive substances to be present in larger quantity in the small intestine and cecum of the germfree mouse and rat than in those of their conventionalized counterparts. One of these substances, designated "musculoactive substance" was observed to be progressively reduced in amount on contamination of germfree rats with a Salmonella typhimurium; concomitant with this, there was a reduction in the size of the cecum. However, after 45 days of exposure, the size of the cecum and the "musculoactive substance" returned to about two-thirds of that found in the germfree animal. Nevertheless, at this time, the numbers of S. typhimurium in the ceca remained elevated. These investigators speculate that the presence of specific bacterial antibody at 45 days, but not earlier, suggests that this immunological response while protecting the monocontaminated host against infection "...places the animal in a carrier state - a state which allows the persistence of high numbers of S. typhimurium in the intestinal tract, but permits the animal to re-assume the characteristics of germ-freeness." Certainly, this is an interesting phenomenon which requires further investigation.

Studies are planned to effect successive passages of cecal flora from ex-germfree (conventionalized) mice to germfree mice in an attempt to "simplify" the flora by this means, and to determine the number of such passages which still allows the evocation of the cecal-reducing and other conventionalizing actions of the original contaminating flora. Qualitative evaluation of the microbial components with each passage should provide the sequential pattern of their alteration, if any, and hopefully, allow the titration of the simplest definable complement of bacteria that will effectively conventionalize germfree rodents.

Germfree Primate: African Green Monkey

A male African green monkey was derived germfree by Caesarean section. The pregnancy was discovered late in the third trimester and the estimated date of delivery determined. Three days prior to the estimated delivery date, the mother was anesthetized with sodium pentothal and halothane. The abdomen was shaved, prepared with soap and tincture of Wescodyne, and sprayed with Vihesive. She was placed in the lower half of a steel surgical isolator (Reyniers ROPU 500) and the abdomen was raised to a Mylar film drape. A lower uterine segment Caesarean section was performed and an active 329g male monkey was delivered. Breathing was immediate. As soon as it was clear that no resuscitation was necessary, it was transferred into a plastic holding isolator.

The monkey was transferred to the Bionetics Research Laboratory, Falls Church, Virginia, where it was kept for 3 months. It was started on 3 ml of formula every two hours. The amount and interval between feedings were progressively increased. The monkey's diet consisted of Enfamil supplemented with 2 drops of Visyneral per day. For the first two and one-half months, it was hand-fed from a baby bottle with a nipple at regular intervals. Thereafter, it was allowed to take milk ad libitum from a bottle with a metal spout.

The monkey was confined to a 10x20x14-inch steel and plastic cage within the plastic isolator for 9 months for its own protection and that of the isolator. The bedding was cloth diapers which were changed 4 times per day. These were later replaced with disposable paper diapers.

No evidence of illness was seen in the monkey. The birth weight was doubled in 98 days, tripled in 182 days, and quadrupled in 280 days. It has remained active with good coordination.

The monkey was free from bacteria and viruses for the first 3.5 months of life. At that time, it was gradually exposed to a bacterial flora. The hematologic and serum protein responses are shown in the following table:

Age, Days	Microbial Flora	Hct, %	Total WBC, mm^3	Poly's, %	Lymph's, %	Total Protein, g	Alb., %	Gamma Glob., %
110	Germfree	46	----	42	55	---	---	---
125	Germfree	47	4,400	46	52	5.4	73	3.0
138	Rhodotorula	50	12,100	47	52	5.9	68	4.0
181	Streptococcus faecalis, E. coli, N. sicca, B. cereus, S. albus	51	10,769	38	59	5.8	67	4.5
197	Heat-killed B. subtilis, E. coli, Proteus, S. faecalis, p.o., then live cultures of these*	--	7,350	13	83	6.2	65	5.0
226	Same plus Rhizopus nigricans and Aspergillus sp.	47	7,128	23	73	6.8	63	6.0

*Isolated from feces of a conventional adult African green monkey.

The following are protein fractions of serum of germfree, ex-germfree, conventional adult monkey, and normal serum standard:

	Total Protein, g	Alb., %	α_1 %	$\alpha_1 A$ %	α_2 %	β_1 %	β_2 %	γ %
Human	7.0	60	6	0	9	8	4	13
Adult Monkey	7.0	46	9	8	16	3	4	14
GF Monkey (125 days)	5.4	73	0	9	11	2	2	3
Ex-GF Monkey (226 days)	6.8	63	0	12	13	2	4	6

The protein fractions of the conventional monkey serum showed, like the human standard, and unlike the germfree monkey, a distinct alpha$_1$ band on agar gel electrophoresis. Like the germfree monkey, and unlike the human, however, a distinct band was seen in the adult monkey between alpha$_1$ and alpha$_2$ which is designated alpha$_1$A. The presumptive interpretation of the alpha$_1$A is that it is the result of microbial contamination, and that the alpha$_1$A is a species specific protein. The gamma globulin increased progressively after contamination, but was still about half that of the adult conventional monkey. The protein electrophoretic patterns were done in collaboration with Dr. N. Papadopoulos of the Department of Biological Chemistry, WRAIR.

The white blood cell count showed an early rise from 4,400 to 12,100, without a pronounced shift to the left. This, however, returned to the 5,000 to 10,000 range and remained so. Hematocrit remained at a "normal" level.

In summary, a monkey was derived germfree by Caesarean section with the standard methods of germfree derivation used in this laboratory. For 3.5 months, it was maintained free from bacteria and viruses. The diet was a simple, commercially available infant formula with vitamin supplement. The monkey was in good condition, his white blood cell count was 4,400, and gamma globulin was only 3% of the total protein. At the age of 3.5 months, it was gradually and progressively contaminated with a microbial flora. The monkey tolerated this well and is continuing in good health.

Hemorrhagic Shock in the Monkey: Effect of 10% Low Molecular Weight Dextran As A Blood Substitute

To test the efficacy of 10% Low Molecular Weight Dextran in isotonic saline (10% LMWD) as a blood substitute after severe, prolonged hemorrhagic shock, African green monkeys were allowed to bleed into an elevated reservoir against a hydrostatic counterpressure of 35 mm Hg for 8 hours. At this time,

the shed blood or an equivalent volume of 10% LMWD was infused. The monkeys of the two treatment groups endured the severe hypotension equally well with small uptake volumes. However, 10% LMWD was far less effective in raising arterial blood pressure, in sustaining it in the early post-treatment period, and in promoting ultimate survival. It also produced an anemia disproportionate to the volume of fluid infused, suggesting hemodilution as a result of the infusion of this hyperoncotic solution. Since one of the goals of fluid therapy for hemorrhagic shock is the rehydration of the depleted extravascular compartment, not its further dehydration, the use of this hyperoncotic solution seems inappropriate.

We cannot preclude that the infusion of 10% LMWD or other concentration of low molecular weight dextran in different volume, route, rate, species, or at other stages of shock might not be effective therapy. We do conclude that 10% LMWD infused into the African green monkey after severe, protracted hemorrhagic shock is not an effective blood substitute. This conclusion bears out the warnings previously made regarding the use of this hyperoncotic solution when a state of dehydration is present. Severe, prolonged hemorrhagic shock is such a state. A detailed report has been submitted for publication.

Peritonitis: Fecal Soilage From a Leaking Suture Line in the Cecum of Germfree Rats

The major portion of the cecum of 19 germfree rats was resected and the thin-walled cecal remnant closed with a single continuous inverting 6-0 silk suture. All recovered from anesthesia. Two were dead at 24 hours, 2 more were dead at 4 days and 2 more at 5 days after surgery. The overall mortality was 32%. At autopsy, generalized fecal peritonitis from a leaking suture line was seen in all 6 rats. The 13 survivors remained well and gained weight at the same rate as germfree rats which had undergone sham-cecectomy. When the survivors were examined 6 weeks later, the cecum was well healed, and there was no evidence of any previous fecal peritonitis. It is concluded that continuing soilage of the peritoneal cavity with germfree feces in the absence of any possible secondary infection of the damaged peritoneal surface is highly lethal. Infecting fecal bacteria may play an important role in conventional animals, but their action would appear not to be necessary for lethality. Further studies are in progress.

Bowel Ischemia Shock: Superior Mesenteric Artery Occlusion (SMAO)

Studies were continued on bowel ischemia shock using the implanted snare device and other methods described in last year's annual progress report from this Department.

Four- to five-month-old germfree and conventionalized Fischer rats were compared. All surgery was performed in a steel surgical isolator (Reyniers ROPU 500) using a system for halothane anesthesia described elsewhere in this report.

The following results were obtained:

Duration of SMAO, Hrs.	No. Rats	Mortality, %	Survival Time, Hrs. After Occlusion Mean±S.E. of Mean	Range	After Release Mean±S.E. of Mean
			GERMFREE		
1.5	10	80	2.1±0.1	1.7-2.8	0.6±0.1
2.0	9	55	2.4±0.1	2.3-2.5	0.4±0.1
3.0	11*	90	3.4±0.2	3.0-4.1	0.4±0.2
Ligated	10	100	4.5±0.4	2.5-6.5	---
			CONVENTIONALIZED		
1.5	14	50	5.0±0.5	2.1-15.0	3.5±0.5
2.0	9**	71	12.2±7.7	2.0-43.0	10.2±7.7
3.0	10	90	6.5±2.2	3.0-25.0	3.5±2.2
Ligated	11	100	5.0±1.9	0.8-10.0	---

*One of these died 2.8 hrs. after SMAO, excluded from calculations.
**Two of these died 1.5 and 1.8 hrs. after SMAO, excluded from calculations.

Mortality after equal periods of SMAO was not significantly different statistically. Mean survival time of the germfree rats was consistently less than that of conventionalized rats. The difference was statistically significant ($p < 0.1\%$) after the 1.5-hour SMAO. Survival time <u>after release</u> of 1.5, 2.0 or 3.0-hour temporary SMAO is also presented to demonstrate the uniformly rapid demise of the germfree rats in contrast to the more varied duration of survival of the conventionalized rats.

At autopsy, the small bowel of all non-surviving rats, except one germfree rat with a 3.0-hour occlusion, appeared cyanotic and congested over 50-90% of its length. The lower half was always involved. Extent correlated with duration of survival. The germfree rats also had a cyanotic cecum. Survivors sacrificed 48 hours after occlusion showed petechiae in the mesentery, but a normal-appearing bowel.

Saline therapy was given to germfree and conventional rats i.p. in a volume equal to 30% of the body weight within 15 minutes of the initiation of a 2.0-hour occlusion. The saline was pre-warmed to 34°C.

The following results were obtained:

Group	Germfree No.	Germfree Mort., %	Germfree Surv. Time Hrs.	Conventionalized No.	Conventionalized Mort., %	Conventionalized Surv. Time, Hrs.
Untreated	9	55	2.4±0.1	9*	71	12.2±7.7
Treated	9	67	23.9±8.1	9	33	22.3±9.6

*Two of these died 1.5 and 1.8 hours after SMAO; excluded from calculations.

Although mortality was not decreased by massive saline therapy, survival time was significantly ($p<0.1\%$) prolonged. At autopsy, the bowel of non-survivors appeared as described above, except in two germfree rats in which only a 2 to 4 cm segment of ileum and patches of the cecum were cyanotic. In contrast to the untreated animals which rapidly became weak and unresponsive after SMAO release, those treated with saline showed relatively little change in behavior after SMAO release.

In summary, no marked differences between the course of SMAO in germfree and conventionalized rats were demonstrated. Some of these observations were presented to the Clinical Congress of the American College of Surgeons on 5 October 1964 in Chicago, Ill. The results are discussed in a manuscript submitted to the OTSG, 4 May 1965.

Limb Ischemia (Tourniquet) Shock

For reasons outlined in our Annual Progress Report of 1964, we have continued study of tourniquet shock in germfree, monocontaminated and conventionalized mice when untreated or given saline. In addition, we have initiated study of 10% Low Molecular Weight Dextran as compared with saline therapy in open-room conventional mice subjected to tourniquet shock.

"Saline-Reversible" Tourniquet Shock - Mice monocontaminated with the yeast Rhodotorula have a gastrointestinal tract that is morphologically indistinguishable from that of germfree mice. A group of such ICR mice, males and females, were randomly selected and divided into two groups. One group remained monocontaminated and the other group was conventionalized. Three weeks after conventionalization, all of the mice were subjected to 3 hours of bilateral hind-limb ischemia by the technique we previously described (Ann. Prog. Rept. 1964). These procedures were performed within a small two-man plastic isolator connected to the animal-holding isolator, as were all other tourniquet studies that follow.

On release of the tourniquets, half of the mice of each group were given isotonic saline, i.p., amounting to 15% of their body weight; the others were untreated. The mice were deprived of food and water for 48 hours after injury. Survivors were sacrificed and non-survivors were examined grossly and preserved for subsequent histopathologic examination. The following results were obtained:

Microbial Status	No. Mice	Treatment*	Mortality, % Hrs. Post-"T" Release 6	12	24	48
Monocontaminated (Rhodotorula)	13	15% B.W. Saline	0	0	0	15
	13	None	0	8	85	92
Conventionalized	9	15% B.W. Saline	0	0	0	11
	9	None	0	44	89	100

*Given i.p. immediately on tourniquet release.

There was no difference in response to this injury between the yeast monocontaminated or conventionalized (ex-monocontaminated) mice, whether treated or untreated. Saline proved effective therapy in both groups of mice.

"Saline-Irreversible" Tourniquet Shock - As described in last year's Annual Progress Report, we have been able to standardize in conventional mice a quadrilateral-tourniquet injury that is unresponsive to saline therapy, i.e., to twice the volume of saline ordinarily found effective in mice when only two of their limbs have been rendered ischemic. Extending such experiments to younger animals this year, 3-month-old male and female ICR mice, either germfree or conventionalized, were subjected to a 3-hour episode of limb ischemia by application of rubber band tourniquets to all four legs as previously described. On removal of the tourniquets, mice were given isotonic saline, i.p., amounting in volume to 30% of their body weight or were left untreated. Food and water were not given for 48 hours after tourniquet release.

The following results were obtained:

"Saline-Irreversible" Quadrilateral Tourniquet Shock

Microbial Status	No. Mice	Treatment*	Mortality, % Post-"T" Release Hrs. 6	18	24	48
Germfree	10	30% B.W. Saline	30	100	100	100
	14	None	100	100	100	100
Conventionalized	14	30% B.W. Saline	35	79	79	93
	16	None	100	100	100	100

*Given i.p. immediately on tourniquet release.

Regardless of saline therapy, there was no demonstrable difference between germfree and conventionalized mice. As compared with older mice (Ann. Prog. Rept. 1964), these younger mice benefitted somewhat less from the saline therapy and died more quickly when untreated. Pooling all the data obtained thus far, gives the following results:

"Saline-Irreversible" Quadrilateral Tourniquet Shock

Microbial Status	No. Mice	Treatment*	Mortality, % Post-"T" Release Hrs. 6	18	24	48
Germfree	32	30% B.W. Saline	19	62	72	81
	28	None	96	100	100	100
Conventionalized	28	30% B.W. Saline	25	64	68	82
	22	None	95	100	100	100

*Given i.p. immediately on tourniquet release.

In summary, the preliminary studies with "saline-reversible" tourniquet shock and the more extensive studies with "saline-irreversible" tourniquet shock have not revealed a difference in survival response among the germfree, yeast monocontaminated and conventionalized mice, whether treated or untreated with saline. As yet, we are unable to explain the waning of the beneficial effects of saline therapy over 48 hours post-injury that occurs when we double the ischemic injury

from 2 to 4 limbs. It is evident that the "doubling of saline therapy" (30% of the B.W.) to offset the "doubling of injury" (tourniquets to all 4 limbs) is equally ineffective, regardless of the microbial status of the mouse. The volume of saline administered to the mice receiving the 4-leg tourniquets would appear to be more than sufficient to overcome any fluid deficit they incur. While the failure of massive saline therapy remains unexplained, it is evident that bacteria are not responsible for the therapeutic refractoriness of mice subjected to ischemia of all 4 limbs for 3 hours. It is noteworthy, that when the tourniquets are left in place for 48 hours, death does not result in either germfree or conventionalized mice. Further studies are planned to investigate this problem.

10% Low Molecular Weight Dextran (10% LMWD) Therapy in Tourniquet Shock - Elsewhere in this report, we have presented observations that hyperoncotic 10% LMWD in 0.9% saline is not an effective blood substitute for the African green monkey after it has endured severe, prolonged hemorrhagic shock. Because one of the primary objectives of fluid replacement therapy of shock secondary to blood or plasma loss is to rehydrate rather than to further dehydrate tissues already depleted of fluid, we considered that therapy for shock with hyperoncotic solution is inappropriate. To investigate this further, we injected intact mice, i.p., with an amount of 10% LMWD in 0.9% saline (Rheomacrodex, Pharmacia) equal to 30% of their body weight, knowing that this amount of isotonic saline is not harmful. We found all 10% LMWD-treated mice to undergo a marked diuresis and to suffer no other overt harmful effect. Learning that hyperoncotic 10% LMWD was well-tolerated when administered i.p. in massive amount to intact mice, the following study was performed:

Open-room conventional male ICR mice, 4-5 weeks old, were subjected to our standard 3-hour bilateral hind-limb tourniquet procedure and housed individually in compartmentalized jars. Ninety minutes after the tourniquets were removed, when significant fluid loss into the injured limbs was in evidence by their swollen appearance, the mice were divided into 3 groups as regards treatment: One group was given an amount of 0.9% saline i.p. amounting to 30% of their body weight; another group similarly received a corresponding volume of 10% LMWD; and the last group received a sham injection, i.e., no treatment other than i.p. insertion of a hypodermic needle. Both the 0.9% saline and the 10% LMWD were at room temperature when administered. Mice from the three treatment groups were handled in alternating sequence as always. Food and water were allowed ad libitum before, but not for 48 hours after tourniquet injury.

The following results were obtained:

Treatment*	No. Mice	Mean B.W. g	Mortality, % Hrs. Post-"T" Release 6	12	24	48	72
None	20	25.0	0	20	65	85	95
10% LMWD, in 0.9% Saline	20	24.9	0	0	15	90	90
0.9% Saline	19	24.4	0	0	11	16	16

*30% of the body weight given i.p. 90 minutes after release of 3-hour bilateral hind-limb tourniquets.

The untreated injury was highly lethal. Saline therapy was beneficial in preventing death throughout the period of observation. 10% LMWD therapy gave significant protection for 24 hours, but by 48 hours all benefit was lost and the mortality of these mice was subsequently no different from that of the untreated mice. Between 24 and 48 hours, the 10% LMWD-treated mice manifested a moderate body tremor. On post-mortem examination, the 10% LMWD-treated mice revealed a large amount of fluid within the peritoneal cavity, which was occasionally red-tinged. A few of the 10% LMWD-treated mice also had focal hemorrhages in the wall of the small intestine.

Although the African green monkeys (see elsewhere in this report) all died when 10% LMWD was substituted for whole blood after prolonged, severe hemorrhagic shock, the duration of their survival after 10% LMWD treatment was apparently not shortened despite the severe anemia they incurred. In the mouse study, we were not sure that 10% LMWD therapy would not reduce survival time below that of the untreated animals. Evidently, 10% LMWD prolonged survival, but this treatment was much inferior to isotonic saline; this appears to relate, in part, to the retention of fluid in the peritoneal cavity of the mice treated with 10% LMWD. Thus, unlike the intact state, the state of shock and the attendant dehydration of tissues that follow tourniquet injury allow demonstration of the potentially deleterious effects of hyperoncotic therapy. This tends to bear out our contention in the monkey study on hemorrhagic shock. It remains to be determined how important the NaCl and water per se in the 10% LMWD solution were in delaying death of the tourniquet-shocked mice. Further work along similar lines is planned to evaluate 10% LMWD in 5% dextrose, which is also commercially available, as compared with 10% LMWD in 0.9% NaCl.

Pathology After Tourniquet Shock - In mice dying of tourniquet shock, gross lesions were confined to the cecum. Yeast monocontaminated mice (with a germfree-like cecal histology) and germfree mice showed cecal hemorrhages, some submucosal edema, and early mucosal ulceration. In addition, clear-cut infarction of the cecal wall was noted in one non-surviving monocontaminated mouse that lived 49 hours. These changes are reminiscent of those in rats subjected to superior mesenteric artery occlusion (see elsewhere in report), in which cecal lesions occurred predominantly in the germfree group. In both situations, the common denominator appears to be a reduced blood flow to the gut during shock. The ceca of the conventionalized mice dying of tourniquet shock were normal.

The "toxic" and "musculoactive" factors demonstrated by Gordon (Nature, 205:571, 1965) to be present in much greater quantity in the small intestine and cecum of the germfree mouse and rat than in those of their conventional counterparts may bear on this. Also, as presented elsewhere in this report, differences in serotonin and histamine content of the cecum between germfree and conventionalized mice may be important in the production of cecal lesions during shock in the former animals.

Burns

As previously outlined, our objective is to study the extent to which bacterial factors influence early mortality (shock phase), delayed mortality and convalescence after burns alone or when combined with ordinarily low-lethal x-irradiation. We have performed additional experiments since last year to compare further the survival response of saline-treated and untreated germfree and conventionalized mice to scald-injury and have made preliminary, incidental observations on the histopathologic alterations of the burn wound. In these initial studies, we have intentionally excluded the genus Pseudomonas as a possible complicating pathogenic factor. Specific studies to evaluate the role of this bacterium alone or combined with others are planned.

Three separate series of experiments were performed, using mice of the same age in each experiment. We concurrently compared the survival of germfree and conventionalized ICR mice, of either sex, after their subjection to a back burn induced by hot water. The conventionalized mice were all former germfree mice that were purposely contaminated with the cecal contents of "pathogen-free" mice of the WRAMC animal colony. The mixed microbial flora of this cecal content was screened in advance to insure exclusion of the genus Pseudomonas which was demonstrated to be absent throughout each experiment. The conventionalized mice from the time of burning had a fecal flora of E. coli, S. faecalis, Proteus sp., Aerobacter and Staphylococcus albus. Weekly cultures were taken of both germfree mice and their conventionalized counterparts to insure that the germfree mice were still germfree and that the conventionalized mice still had the aforementioned flora and were still Pseudomonas-free; cultures were also taken for the same purpose at the end of each experiment, as always. Other than for the presence of the aforementioned flora, the

conventionalized mice were housed, maintained and handled in every respect identical to that of the germfree mice. The ex-germfree mice were not used in any experiment until at least 3 weeks had elapsed from the time they were contaminated.

The burn procedure, as were all other procedures, was performed within the isolators. A special small, two-man isolator (see Ann. Prog. Rept., 1964) connected to the animal-holding isolator was used for this purpose. Mice, after anesthesia (40 mg/Kg pentobarbital i.p.), were passed singly to the two-man isolator for burning, and were transferred back to their holding-isolator before the next mouse was burned. The Bronwill Constant Temperature Circulator (Will Corp.) was used to maintain the desired water temperature. The mice were scalded at 73°C. for 10 seconds in a stainless steel receptacle containing 6 liters of water to which 5 ml of Tween 80 had been added. The latter served to insure access of the hot-water to the skin of the non-depilated dorsum of the mouse. The burn covered 30-40% of the body surface area, the dorsum of the mouse having been immersed to the mid-axillary line from the base of the neck to the base of the tail. The mouse was then immediately and identically immersed in ambient temperature water (25-27°C.) for 10 seconds to halt the burn process, and thereby inflict a more reproducible injury. Immediately after this, the mice were alternately either given a single i.p. injection of isotonic saline amounting to 15% of their body weight or were left untreated. The mice were then promptly returned to their holding-isolator where they were gently patted dry. Food (autoclaved L-356 diet) and water were allowed ad libitum throughout the entire course of the experiment, pre- and post-burning. Mice that died during the 4-week observation period were examined grossly and preserved in formalin, as were the survivors at sacrifice. Below are the pooled results from all 3 experiments.

Microbial Status	Treatment	No. Mice	Mortality, % Days Post-Burn 0.25	1	2	3	7	14	28
Germfree	None	22 M, 22 F	2	23	30	45	55	61	61
Conventionalized (Pseudomonas-free)	None	21 M, 21 F	2	10	43	52	55	57	60
Germfree	15% B.W. Saline i.p.	24 M, 21 F	4	9	13	16	18	22	24
Conventionalized (Pseudomonas-free)	15% B.W. Saline i.p.	21 M, 20 F	0	0	2	5	5	7	10

As we anticipated, the burn resulted in an approximate LD_{50} in the untreated conventionalized mice by 72 hours. It is evident that whether saline-treated or not, the conventionalized mice did not fare worse than the germfree, either during the first 72 hours or later post-burn. Whereas a statistically greater survival was evident by 48 hours for the conventionalized mice given saline as compared with those untreated, statistical evidence of this was delayed in the saline-treated germfree mice until 72 hours post-burn.

With this inflicted burn, there were few delayed deaths beyond 72 hours during the remainder of the 4 weeks of observation. However, it is noteworthy that delayed deaths did occur in the saline-treated and untreated mice of the germfree as well as the conventionalized groups. The mechanism(s) behind the delayed death of the burned germfree mouse is of special interest, but presently unknown.

Burn Wound Histopathology - During the first 96 hours post-burn, no difference in the extent or degree of the burn could be determined between the germfree and conventionalized mice. Deep second to third degree burn changes were present. In the burn zone, all but a few of the very deepest hair follicles were damaged. The swelling and alteration of the staining properties of the dermal collagen were variable. During this period, there was never more than a trace of an inflammatory reaction. The superficial deep muscle below the dermis showed minimal damage in both groups of mice.

At two weeks post-burn, most of the burn lesions appeared to be third degree with eschar or open ulcer showing early epithelial regeneration at the edges. On the surface of the wound, considerable acute inflammatory reaction was seen, more so in the conventionalized mice in which bacterial colonies were present. The extent of the burn and early healing were the same in the germfree and conventionalized mice. Both showed considerable granulation tissue and collagen deposition in the injured area. The major difference between the burned areas of the germfree and conventionalized mice related to the greater inflammatory reaction and the presence of superficial colonies of bacteria in the latter animals.

By four weeks post-burn, the inflammatory reaction was equally marked in both groups where healing had not been completed. Superficial infection of the burn zone was present in some of the conventionalized mice. However, the degree of healing was somewhat greater in the conventionalized mice, residual ulcers and eschar being smaller and less frequent than in the germfree. This is an interesting finding, but more detailed observations are necessary to confirm this initial impression.

Further comparative studies are in progress or are planned to investigate: the effect of specific bacterial contamination of germfree mice with bacteria considered or known to be pathogenic after burns and the efficacy of antibiotics to which the bacteria are sensitive; the effects of more severe burns, with and without therapy; the effects of burns produced by radiant heat rather than hot water; the use of halothane instead of pentobarbital anesthesia; burns in rats; and more detailed sequential histopathologic observations on the evolution of the burn wound and its healing.

Uremia: Effect of the Microbial Flora

Further studies were carried out on germfree, conventionalized, and defined-flora animals in order to determine the importance of the microbial flora.

Five groups of 6- to 7-month-old Fischer rats were studied. All were obtained germfree and maintained in isolators throughout the experiment. Fifty ml of thioglycollate broth were added to their food, water, and bedding twice a week for 4 weeks prior to surgery. The broth contained the following:

Group	Microbial Status	Broth
1	Germfree	Sterile
2	Monocontaminated	48 Hr. culture of S. albus
3	Dicontaminated	48 Hr. culture of: S. albus Proteus mirabilis
4	Tetracontaminated	48 Hr. culture of: S. albus Proteus mirabilis E. coli Streptococcus faecalis
5	Conventionalized	Sterile

The rats in Group 5 were germfree until 2 months prior to surgery. At that time, they were conventionalized with the cecal contents of rats from the WRAMC "pathogen-free" colony. The only organisms growing aerobically were those of Group 4. Anaerobes were present but were not identified. Fecal material was obtained at weekly intervals and cultured on blood, MacConkey's and Sabouraud's agars and in thioglycollate broth to insure that the bacterial flora was as described. The organisms in Groups 2, 3, and 4 were originally isolated from the cecal contents of WRAMC "pathogen-free" rats.

The rats received steam-sterilized, semi-synthetic L-356 diet and water until the day prior to surgery. At that time, they were allowed only 5% dextrose in 0.9% NaCl. Postoperatively, they were allowed no food or water.

Bilateral nephrectomy was performed under halothane anesthesia via an abdominal approach. Postoperatively, they were individually housed on wire screen and observed until death. Fasting controls were observed for 7 days at which time all were sacrificed with CO_2.

The following results were obtained:

Nephrectomized

Group	Microb. Status	No. of Rats	Body Weight, g	Survival Time, Hrs.	Observed Range, Hrs.
1	GF	6	269±7	122.2±6.2	90-139
2	Mono-	10	188±10	112.2±8.9	60-135
3	Di-	9	189±14	109.2±3.4	89-119
4	Tetra-	8	188±11	90.5±8.1*	57-128
5	Conv	11	195±17	75.9±4.3**	41-95

Mean±S.E. of mean.
*Significantly less than germfree, $p < 1\%$.
**Significantly less than germfree, $p < 0.1\%$.

Controls

Group	Microb. Status	No. of Rats	Initial Body Wt., g	% Body Wt. Loss at 7 Days	Cecal Wt., % Final Body Wt.
1	GF	2	268	31.1	5.7
2	Mono-	6	192±18	30.1±1.2	9.0±1.6
3	Di-	6	182±13	27.3±1.2	6.3±0.6
4	Tetra-	6	182±15	25.4±2.9	8.8±1.0
5	Conv	6	197±22	26.3±1.1	0.6±0.1

Mean±S.E. of mean.

The mean survival time decreased with the increased complexity of the bacterial flora. A significant difference was shown between the germfree and tetracontaminated rats, and previous observations (Ann. Prog. Rept. 1964) showing the shorter survival time of conventionalized rats compared to germfree rats were borne out.

All controls lived 7 days. Weight loss was at approximately the same rate. The cecal weight of the defined-flora animals was no different from that of the germfree.

No gross infection or other lesions were seen at autopsy. Histologically, all groups showed myocardial necrosis and calcification. The cecal ulcers observed by us in previous work were found again only in the conventionalized rats dying of uremia in Group 5. They were not found in the controls of Group 5 or in any of the rats of Groups 1 to 4.

Further observations were also made on the chemical composition of the blood, muscle, and cecal contents of rats. Germfree and conventionalized rats were nephrectomized or sham-nephrectomized as above and deprived of food and water. Seventy-two hours later, they were heparinized (30mg/Kg i.m.) and anesthetized with halothane. The abdominal aorta was cannulated and the rats exsanguinated. The blood, plasma, muscle and cecal contents were analyzed. The following results were obtained:

	Sham-Nephrectomy		Nephrectomy	
Variable	Germfree	Conv.	Germfree	Conv.
Hematocrit, %	45.0±1.4* (8)	45.6±2.9* (8)	28.8±1.4 (12)	33.2±1.7 (11)
Blood Urea N, mg%	51.8±5.4* (8)	33.0±6.8 (8)	334.8±14.8 (12)	398.1±20.6 (11)
Cecal Content Urea N, mg%	110.8±15.3* (8)	101.7±16.5 (7)	319.1±25.8 ** (12)	102.1±24.8 (10)
Plasma K, mEq/L	4.2±1.2* (8)	3.6±0.1* (7)	7.9±0.3 (12)	8.4±0.2 (9)
Muscle K, mEq/Kg	115.5±6.0 (8)	104.7±6.0 (7)	128.5±6.0 (12)	116.6±2.5 (11)
Muscle Na, mEq/Kg	28.7±1.8 (8)	27.7±3.9 (7)	31.2±1.4 (12)	26.3±2.5 (11)
Plasma PO_4, mEq/L	3.0±0.5* (8)	3.6±0.4* (8)	9.5±0.7 (12)	9.0±0.9 (11)
Plasma Ca, mEq/L	4.9±0.1 ** (8)	6.9±0.5 (8)	5.4±0.3 (12)	6.6±0.4 (11)
Plasma Acidic Indole Compounds***, mg%	0.12±0.02 (3)	0.27±0.18* (4)	0.09±0.01 ** (9)	0.54±0.08 (5)

Number of animals in parentheses.

*Sham-Nephrectomy significantly different ($p = 1\%$ or $<$) from Nephrectomy.
**Germfree significantly different ($p = 1\%$ or $<$) from Conv.
***Calculated as indoxyl sulfuric acid.

In response to bilateral nephrectomy, the table shows that an anemia occurs in both germfree and conventional as does a rise in plasma and muscle potassium, and plasma phosphate. Differences between the germfree and conventional response were found in the greater rise in blood urea nitrogen and in "indican" in the conventional rat, and a greater rise in cecal urea nitrogen in the germfree rat.

In summary, the previous observations of the decreased tolerance of the conventionalized rats to uremia were verified and extended to show that a limited number of bacterial species may produce this decreased tolerance. Their mechanism of action remains to be determined by the further studies which are in progress. Manuscripts on the completed phases of this work are being prepared.

Antibiotics: Antibacterial vs. Non-Antibacterial Activity

Chlortetracycline-Induced Fatty Liver and Other Effects - Previous experiments in this laboratory dealing with single or multiple injections of various antibiotics of the Tetracycline group (tetracycline, oxytetracycline, and chlortetracycline) have resulted in fatty metamorphosis of the livers of mice when given in a proper time-dose relationship. Publications by Lepper, et al. (A.M.A. Arch. Int. Med. 88:284, 1951) and Seto and Lepper (Antibiotics and Chemotherapy, IV:666, 1954) involving similar studies have stated that of the three aforementioned antibiotics, chlortetracycline produces the greatest degree of lipogenesis. These findings were corroborated in this laboratory with the use of open-room conventional mice (see Ann. Prog. Rept. 1963).

Monocontaminated mice were then treated similarly (together with conventional controls) and the results of this experiment were reported in last year's Annual Progress Report. A follow-up study was planned and outlined in this report, and the following is a summary of the manipulations and results of this experiment, together with the possible implications of our findings.

Germfree and conventionalized ICR mice of either sex and comparable age (3 months) were used concurrently in this experiment. Approximately half of the mice were given a single i.p. injection of Chlortetracycline HCl (Lederle Labs, Inc.) amounting to 100 mg/Kg (1% solution in saline) per day for 2, 4 or 6 consecutive days. The other mice were given a single i.p. injection of isotonic saline amounting to 10 ml/Kg per day for 2, 4 or 6 consecutive days. (Dosage was based on the body weight at the beginning of the experiment.) Each animal was weighed daily. Food (L-356 diet) and water were given ad libitum throughout the experiment. All procedures, up until the time of sacrifice, were performed within the animals' holding isolators. During the injections, only two mice died. Both were from a group of 5 female conventionalized mice that had received 6 consecutive daily i.p. injections of chlortetracycline. Their deaths occurred after the 6th injection and before they were due for removal and sacrifice; these will be discussed below. At the time of sacrifice, the mice were removed from their isolators and killed by CO_2 inhalation. Blood was immediately withdrawn from the heart. The cecum was removed

in toto and cultured under aseptic conditions. The liver was excised, weighed and sliced into three pieces: one piece for wet-dry weight measurement, another piece for histologic examination and the remainder was frozen in dry ice for lipid analyses. The spleen, small and large intestines were then removed. The cecum, small and large intestines were all split, washed gently in cold isotonic saline, and patted dry. These tissues, along with the spleen, were then weighed in metal pans and frozen in a mixture of alcohol and dry ice. The frozen tissues were placed in a container of HCl and homogenized with a Waring blender. After being brought up to the proper volume, the suspensions were refrigerated at -75°C. for future analyses. One kidney of each animal was heat-sealed in a plastic bag and frozen at -75°C. for fluorescence microscopy; the other was preserved in 10% buffered formalin.

Biochemical and Physiological Observations - The following tables show some parameters of the saline-injected conventionalized and germfree animals which we will refer to as "normal" controls:

			Liver			Small Intestine	
Microb. Status, No.& Sex	Initial Body Wt., g	BUN mg%	Weight, g/100g B.W.	Water %	Non-Fat Solids, g/100g B.W.	Weight, g/100g B.W.	Water %
Conv, 13 M	32.5±1.1	25±4	5.20±0.23	68.1±0.5	1.38±0.06	3.4±0.2	74.5±1.5
						$p < .02$	
GF, 14 M	31.6±0.6	26±3	4.98±0.18	69.5±1.1	1.23±0.03	3.9±0.1	75.9±0.6
Conv, 15 F	27.7±0.9	21±5	4.65±0.13	69.5±1.1	1.15±0.03	4.0±0.1	76.9±0.4
						$p < .01$	
GF, 14 F	27.8±0.6	25±4	4.12±0.10	69.3±0.8	1.08±0.04	4.8±0.1	77.8±0.6

Mean±S.E. of mean.

Normal Liver Lipids*

Microbial Status	No. Mice and Sex	Total Lipids	Glycerides	Total Cholesterol	Phospholipids
		mg/g dry liver			
Conv	13 M	167±10	40±3	12.5±1.1	89±4
		$p < .05$	$p < .1$	$p < .1$	
GF	14 M	139±8	34±2	10.4±0.6	93±5
Conv	15 F	184±7	48±3	17.2±1.4	97±5
		$p < .01$	$p < .05$	$p < .1$	
GF	14 F	148±7	40±2	13.8±1.1	98±5

Mean±S.E. of mean.
*All mice received 2, 4 or 6 injections of isotonic saline (10 ml/Kg i.p.).

Since it is important to determine whether, and to what extent, normal conventional and germfree mice differ with respect to physiologic and anatomic parameters, we have considered them separately from the results of the main experiment. There were no differences between germfree groups of males or females with respect to body weight, blood urea nitrogen, and water contents of liver and small intestine. There was a tendency (not statistically significant) toward heavier livers per unit body weight in the conventional animals than in their germfree counterparts; the same was true for the non-fat solids of the liver. Conventional animals, whether male or female, had consistently, and for the most part, statistically significantly higher concentration of liver lipids, glycerides, and cholesterol than their germfree counterparts. Phospholipids, on the other hand, were equal in conventional and germfree animals of a given sex. The small intestine of the germfree animal was also heavier than that of its conventional counterpart.

Besides these germfree-conventional differences, there were also a number of interesting sex differences (per unit B.W.); the female liver weight per unit body weight was smaller than the males, non-fat liver solids also followed this pattern, while the weight of the female small intestine and all the liver lipids were greater than in the males. These relations held true for both germfree and conventional mice. We will not comment further on these sex differences except to note that one or more of them may be important in explaining differences in reaction to chlortetracycline.

The most dramatic differences in saline-control animals (see tables above) are concerned with the liver, its composition, and the weight of the small intestine. The following tables show that the most dramatic effect of chlortetracycline that we measured occurred precisely within these parameters, and it may be that this antibiotic tends to intensify differences already existent between germfree and conventional mice, and males vs. females. It should also be pointed out that the only animals which died after chlortetracycline treatment were two conventional females; the biochemical determinations afford no obvious explanation of this phenomenon.

Percentage Differences With Respect to Saline Controls*

Microb. Status**	Sex	Body Wt.	BUN	Liver			Small Intestine	
				Weight	Water	Non-Fat Solids	Weight	Water
Conv	M	- 8.9	+108.0	-10.0	- 0.6	-25.4	-22.5	+9.2
			$p < .02$		$p < .01$			
GF	M	-15.6	-69.2	+ 8.2	- 8.8	-19.5	- 8.7	+4.5
Conv	F	- 5.0	+123.8	+12.2	-11.2	+13.9	+ 6.2	+3.3
			$p < .01$					
GF	F	- 2.2	- 28.0	+22.6	- 6.5	+ 8.3	+22.0	+2.4

*Experimental animals received i.p. 100mg/Kg chlortetracycline per day for 4 days. See Table of Normal Mouse Values for units.

**Each group consisted of 9 or 10 animals.

Percentage Differences With Respect to Saline Controls*

Microb. Status**	Sex	No. of Injections 2	4	6	No. of Injections 2	4	6
		Total Lipid			Glycerides		
Conv	M	+ 55.2	+ 87.8	+105.2	+127.6	+172.8	+173.0
		p < .01	p < .05		p < .01	p < .05	
GF	M	+217.5	+230.5	+ 93.1	+432.7	+340.6	+157.2
Conv	F	+ 99.4	+ 82.5		+126.2	+135.8	
GF	F	+127.8	+129.1	+117.6	+185.1	+169.7	+138.0
		Cholesterol			Phospholipids		
Conv	M	+ 16.0	+ 34.2	+ 87.5	+ 71.0	+110.7	+ 84.9
			p < .1	p < .1			
GF	M	+ 58.5	+103.9	+128.0	+171.8	+163.5	+ 64.9
Conv	F	+ 10.9	+ 8.7		+ 76.7	+ 54.4	
			p < .02				
GF	F	+ 42.5	+ 75.9	+ 45.5	+ 79.6	+ 76.2	+ 54.7

*Experimental animals received i.p. 100mg/Kg chlortetracycline per day for 2, 4, or 6 consecutive days. See Table of Normal Mouse Values for units.

**Each group consisted of 9 or 10 animals.

There are no consistent differences in the response of the groups with respect to loss of body weight, although the germfree males lost the most, and the germfree females, the least. All groups gained liver weight except the conventionalized males, a phenomenon undoubtedly related to the relatively mild fatty liver induced in this group with the concomitant large loss of liver (non-fat solids). With respect to loss of liver water, again the germfree male fared worse, while the conventionalized male and germfree female did best. The reaction of the animals with respect to "non-fat liver solids" (protein) seems connected with sex alone. If bacterial status was a factor, it was overridden completely by the sex factor. Changes in BUN, on the other hand, seem dominated by bacterial status; this finding is significant in view of the reported use of tetracycline for treatment of kidney infections in humans and the universal rise in BUN. The possible significance of our findings will be discussed later.

In summary, some of the effects of chlortetracycline are dominated by sex, and others by bacterial status. If loss of water can be accepted as a significant factor in liver status, the germfree male and conventional female fare worse. It would appear from the "non-fat solids" column, that the females, regardless of bacterial status, do not suffer a liver anti-anabolic or catabolic effect, while the males do.

As for the BUN levels, one may conclude either that the germfree mice suffered no renal dysfunction while the conventional mice did (see below), that they were able to excrete urea extra-renally while the conventionals could not, or possibly that microorganisms contribute directly to urea levels.

The greatest contrast in the effect of chlortetracycline on liver lipids occurred between the two male groups, with the females occupying intermediate positions, but the germfree males showed by far the most immediate and greatest lipid increase (percentage-wise) which, however, declined by the 6th injection. The differences between the conventional and germfree males are statistically significant for part of the total lipids and glycerides, and almost so for cholesterol. It appears, furthermore, that the type of lipid underwent a qualitative change for both groups of males, with the cholesterol rising continuously as the glycerides remained stable or, in the case of the germfree males, declining. In general, the germfree females also showed higher lipid levels with respect to their saline controls than did their conventional counterparts, but these differences were not as dramatic as those between the male groups. It will be noted that there are no values for the conventional 6 injection females; deaths in this group reduced the number to only three, insufficient for statistical analysis. There was no glyceride to cholesterol shift as in the males, and the phospholipids were fairly uniformly elevated.

Histamine - Histamine is present in greater concentration in the small intestine of saline-injected germfree females and in the cecum of germfree males as compared with their conventionalized counterparts; there are no other significant differences. After chlortetracycline treatment, however, all gastrointestinal components, however calculated, and in all groups are greatly reduced. The reduction in small intestine histamine appears to exceed the concomitant slight changes in water content of the small intestine of all mice that also follows antibiotic treatment, and, therefore, does not seem explicable on the latter basis. The small intestine of the normal (saline-treated) germfree males and females has a small, but statistically significantly greater fresh weight than that of the corresponding conventional animals. Similarly, water content of the germfree animals' small intestine is slightly higher, but the differences are not statistically significant. After chlortetracycline, females showed largest increases in fresh small intestine weight, and for either sex, the conventional increase slightly exceeded the germfree. (See tables below.)

Serotonin - As with histamine, serotonin tends to be greatest in the normal germfree animal, particularly in the female group. After chlortetracycline treatment, changes are variable, except in the ceca, in which serotonin decreases greatly in the germfree animals of both sexes, while in the conventionals it actually rises. (See tables below.)

In summary, then, it can be said that when significant differences are seen in either histamine or serotonin in the GI tract, they are always in the direction of greater concentration or content in the germfree animal; treatment with chlortetracycline universally lowers the total amount and concentration of these substances in all parts of the germfree GI tract (of both sexes), while its effect on conventional mice is more variable, in the case of serotonin, it may actually cause a rise.

Histopathology of Chlortetracycline-Injected Mice - All the livers of the chlortetracycline-injected animals showed marked increase in fat compared to saline controls. The fat occurred as multiple small vacuoles in the cytoplasm of liver cells in the centrilobular zone of the liver lobule in the less severely affected animals and involved the entire lobule in severe cases. Less frequently, one or two larger globules of fat replaced the cytoplasm. No other type of liver cell degeneration was seen; there was no necrosis of liver cells. Saline animals had no fatty change or a mild fine periportal fatty change with Oil Red O stain.

In the kidneys of the antibiotic-treated groups, a patchy tubular calcification and necrosis was found in the convoluted tubules of the corticomedullary junction and to a lesser extent in the cortex itself. The calcific changes occurred as tiny droplets in necrotic cells, as droplets along an attenuated brush border of the proximal tubules, and as larger aggregates replacing tubular segments. Usually, the changes were not extensive, most tubules being spared.

Normal Mouse* Values for Gut: Serotonin, Histamine, Small Intestine Weight, and Water Content

Microb. Status & Sex**	Total Small Intestine, µg/100g B.W.	Cecum µg/g tissue/100g B.W.	Large Intest. µg/g tissue/100g B.W.	Small Intest. µg/g tissue/100g B.W.
		Serotonin		
Conv, M	33.6±4.7	10.6±1.6	29.8±1.6	33.6±3.9
		p < .01		
GF, M	35.6±1.0	20.0±1.5	22.5±4.3	33.8±3.1
Conv, F	35.6±3.2	9.9±1.0	27.2±1.6	39.2±4.0
	p < .05	p < .01	p < .01	
GF, F	52.0±4.8	20.9±2.0	42.6±3.7	44.9±4.0
		Histamine		
Conv, M	3.0±0.6	5.5±0.4	3.2±0.3	3.1±0.6
		p < .02		
GF, M	2.3±0.5	25.2±6.7	3.5±0.4	2.6±0.2
Conv, F	2.4±0.4	9.2±0.7	5.8±1.2	2.7±0.5
	p < .05			
GF, F	4.1±0.4	7.0±1.7	5.0±0.3	3.6±0.3

Small Intestine

	Weight, g/100g B.W.	Water, %
Conv, M	3.0±0.1	75.6±1.4
	p < .02	
GF, M	3.5±0.1	76.2±0.8
Conv, F	3.5±0.2	76.7±0.7
	p < .02	
GF, F	4.4±0.3	78.2±1.6

*Six injections of saline (10 ml/Kg i.p. daily).
**Each group consisted of 4 to 5 mice.

Percentage Differences in Histamine and Serotonin of GI Tract*

Microb. Status & Sex	Total Small Intestine	Cecum	Large Intest.	Small Intest.
		Serotonin		
Conv, M	-16.6	+ 3.7 $p < .02$	-43.6	-13.4
GF, M	- 7.8	-27.1	-23.6	- 5.6
Conv, F	+34.0	+51.5 $p < .05$	+22.8	- 7.1
GF, F	- 0.8	-18.1	-11.0	+ 1.6
		Histamine		
Conv, M	-26.7	-38.6	-50.0	-29.0
GF, M	-13.1	-72.6	-48.5	- 7.7
Conv, F	-75.0	-43.5	-75.9	-69.2
GF, F	- 7.3	-60.0	-72.0	-25.0

	Small Intestine	
	Weight	Water
Conv, M	+ 3.0	+4.3
GF, M	- 2.9	+1.8
Conv, F	+37.2	-3.1
GF, F	+23.4	-1.0

See Table of Normal Values for units.

*Each animal received 100mg/Kg i.p. chlortetracycline per day for 6 days.

In two conventional females that died on the last day of the experiment (after having received 6 injections of antibiotic), the cortical tubular necrosis was extensive and undoubtedly significantly contributed to their death. One of these had a necrosis of the tip of the renal papilla, and both showed moderate to marked fatty livers, congestion of the lungs and liver, lymphocytic atrophy of the spleen. Only one male of a total of 56 male and female saline controls showed a mild cortical tubular calcification. The table below shows the incidence of cortical tubular calcification in accordance with the number of injections of antibiotic and its severity in the different groups.

Microb. Status	Sex	No. of Injections				
		2	4	6	All,	Severity,*
		No. Positive/Total			% Pos.	Mean Score
Conv	M	0/4	2/5	1/5	21%	1.0+
GF	M	0/5	0/5	0/5	0%	0
Conv	F	3/5	2/5	4/5**	60%	2.2+
GF	F	0/4	1/5	1/5	14%	trace

*The degree of tubular calcification was graded as follows:

trace = few scattered foci (1 to 5) per section.
1+ = ca. 15 scattered foci per section.
2+ = 30 to 40 foci per section.
3+ = 100 to 150 foci; ca. 20% of cortical tubular area involved.

**Two of these mice died after the 6th injection; preserved in formalin.

Tubular calcification may occur spontaneously in some strains of mice, but this appears clearly ruled out by the rarity of such findings in the saline controls. In general, BUN was elevated in cases when calcification was seen, although there were numerous instances of high BUN without observable calcification. Chronic inflammatory changes on the peritoneum of the liver and kidney were minimal to absent in the saline-treated specimens and minimal to mild in the chlortetracycline animals. The germfree male consistently showed the least chronic peritoneal inflammation, but the differences were not striking.

Some questions raised by this chlortetracycline study are: Why did only conventional females die? Why was renal damage seen only in the conventional females and males? What accounts for the differences in the livers of normal germfree and conventional animals and their different responses to chlortetracycline? What accounts

for the shift to abnormally high liver cholesterol levels (from high levels of glycerides) in the males? What accounts for the sharp sex difference (regardless of bacterial status) with respect to gain or loss of liver non-fat solids? What accounts for the sharp bacterial status differences (regardless of sex) with respect to increase or decrease in BUN?

Since fatty infiltration of the liver could be secondary to protein derangements, we feel it is profitable to study liver proteins, particularly those with specific functions, viz., enzymes. Therefore, we have initiated a long range study of some of those liver enzymes which are concerned with protein anabolism and catabolism, and the effect of chlortetracycline, starvation, and the germfree state on these enzymes.

Amino Acid Metabolism

Our last progress report outlined a four-fold approach to the question of the mechanism of d-amino acid inversion in the mammal to the usable l-form, and to the more general problem of amino acid and protein metabolic differences (if any) in the germfree versus the conventional rat. In the last year, we have completed or made substantial progress in three of these approaches, and have begun others as well.

Tryptophane Pyrrolase - We have confirmed our previous findings concerning this enzyme after feeding d- and l-tryptophane to germfree and conventional rats (see last year's annual progress report); furthermore, we have extended our observations to include i.v. administration of tryptophane. It would appear that the rat liver can be induced to elaborate this enzyme *in vivo* equally well by administration of l- or d-tryptophane i.v.; there was no apparent rate difference in this process in the germfree vs. the conventional rats. (The differences seen in the *per os* experiment of last year likely reflect the markedly slower absorption of the d-isomer from the intestine.) Some of the results of this enzyme assay carried out with rat liver homogenates after tryptophane administration are as follows:

Sacrifice Time*, min.	Dose, mg & Route of Rx	Liver Tryptophane Pyrrolase (μM kynurenine formed in vitro/hr/gm dry liver)					
		Saline		l-Tryptophane		d-Tryptophane	
		Conv	GF	Conv	GF	Conv	GF
50	25 i.v.	11.6±2.8	6.8±1.1	24.1±3.5	23.1±3.2	20.3±2.6	19.2±2.9
150	25 i.v.	22.7±4.1	17.3±2.6	38.7±3.5	27.2±2.5	36.9±2.6	31.0±1.6
180	100 p.o.	2.3±0.4	2.0±0.3	48.0±8.7	48.9±2.8	28.5±2.0	22.3±4.5

Mean±S.E. of mean; all males, weighing from 180-220 grams; each group 4-5 rats.
*After tryptophane administration.

In our last progress report, we speculated that the tryptophane pyrrolase rise after d-tryptophane might be hormonally induced. The following table shows that this is almost certainly not so.

Group	No. Rats*	Treatment	Liver Tryptophane Pyrrolase**
Sham-Adx.	3	l-form	35.0
Adx.	1	l-form	27.0
Sham-Adx.	3	d-form	25.0
Adx.	3	d-form	18.5
Sham-Adx.	3	Saline	16.7
Adx.	1	Saline	2.3

*All male conventional rats; each rat received 100 mg l- or d-tryptophane or equal volume of saline i.p.

**µM kynurenine formed in vitro per hr. per gram dry liver.

Groups of animals were adrenalectomized or sham-operated, allowed three days to recover, and then given 100 mg l- or d-tryptophane i.p. D-tryptophane still gave rise to large amounts of the enzyme, even in the adrenalectomized rats. We think this proves that inversion of the d-isomer does occur very rapidly, probably within a few minutes of administration and that adrenal mechanisms are not required.

The rapidity and power of this inversion is very striking. We will show, in the following paragraphs, that there is probably a rate difference in this reaction in germfree as against conventional rats despite the fact that this difference cannot be demonstrated by the "tryptophane pyrrolase induction" method.

$C^{14}O_2$ - Figure 1 shows the cumulative $C^{14}O_2$ expiration after i.v. administration of 5µc of C^{14} l-tryptophane to germfree and conventional male rats; CO_2 samples were taken every 10 minutes for the first two hours, every 20 minutes thereafter, and assayed for C^{14} by scintillation counting; the bars represent standard errors of the mean. The germfree animals expired significantly more $C^{14}O_2$, until there was a $2\frac{1}{2}$-fold difference by the fourth hour, at which time the experiment was terminated. This greater and apparently ever-increasing $C^{14}O_2$ expiration difference occurred despite the somewhat greater body weights of the conventional animals (302 grams mean vs. 250 grams), and also greater conventional mean liver weights (10.34 grams vs. 8.19 grams). Thus, it appears that the germfree rat metabolizes at least part of the carbon skeleton of the l-tryptophane at a significantly

faster rate than the conventional rat does. This finding should have significance in the elucidation of the involvement of bacteria in mammalian amino acid and protein metabolism.

Figure 2 shows the results of the same type of experiment carried out with C^{14} d-tryptophane. In this experiment, mean body weights (conventional vs. germfree) were 204 grams vs. 226 grams, and liver weights were 6.30 grams vs. 6.82 grams. The results of this experiment were precisely the opposite of the preceding one, that is, the conventional rats expired more $C^{14}O_2$ than the germfree in 4 hours rather than less (although the figures were not statistically significant). Furthermore, at the end of 4 hours, this group of conventional rats had expired twice as much $C^{14}O_2$ as those which had received the l-tryptophane.

In summary, the process by which $C^{14}O_2$ is expired after C^{14} d-tryptophane i.v. administration seems fundamentally different than that after C^{14} l-tryptophane, since, in the latter case, an "s"-shaped curve is observed, while in the former, the process seems quite, if not perfectly, linear; it appears as though the l-tryptophane is more rapidly and efficiently converted to CO_2 by the germfree rat, while the opposite holds true for the conventional. We tentatively conclude that the inversion process is more efficient in the conventional animal, and we are pursuing this hypothesis.

<u>C^{14} Uptake by Tissue Proteins After C^{14} Tryptophane Administration</u> - C^{14} uptake by proteins of liver, small intestine, large intestine and cecum was measured by scintillation counting of the cold TCA precipitable material (subsequently digested in 2M KOH) after i.v. administration of 5μc of l-tryptophane. The results are presented in Figures 3 and 4; cecal content of C^{14} is also shown in these two figures.

Considering first the l-tryptophane, it would appear that the germfree rat incorporates larger amounts of C^{14} into the protein of rapidly metabolizing tissues (liver and small intestine) up to 24 hours after administration than the conventional animal does. With respect to the large bowel and cecum, the differences are not as consistent, although the trend at earlier hours is the same as that of the liver and small intestine. Germfree rats also had consistently more C^{14} in the cecal contents. Thus, the tissue protein uptake parallels the $C^{14}O_2$ expiration; the carbon skeleton is more rapidly catabolized and incorporated into protein of the germfree rat.

Figure 4 shows results of the same type of experiment carried out with C^{14} d-tryptophane. Here, the protein bound C^{14} of liver and small intestine tends to be higher in the conventional rat. This again parallels results with CO_2 analyses. The large intestine and cecum incorporations are variable, although they tend to be higher in the germfree at the 4-hour point. At 24 hours, the cecal content of the germfree contains 7 times as much C^{14} as that of the conventional.

It appears that with respect to the rapidly metabolizing tissues, the germfree rat incorporated C^{14} most rapidly from l-tryptophane, and the conventional, from d-tryptophane. This might indicate a basic advantage of the germfree toward l-tryptophane metabolism and a defect in the d- to l- inversion process. The following table summarizes the data without regard to times after administration:

No. Rats/ Group	Tissue, etc.	% of Injected Dose C^{14} Incorp./g Protein[a] GF	CONV	% Difference[c]
		C^{14} l-form injected		
8	Liver	7.0	5.5	+ 27
8	Small Intest.	10.1	6.8	+ 48
6	Large Intest.	2.8	2.5	+ 12
6	Cecum	4.0	3.4	+ 18
6	Cecal Content	1.4	0.7	+100
		C^{14} d-form injected		
6	Liver	4.9	5.0	- 20
5	Small Intest.	7.9	9.2	- 14
5	Large Intest.	3.6	3.6	0
5	Cecum	4.5	3.6	+ 25
5	Cecal Content[b]	1.8	0.9	+100

[a]Regardless of time after administration of sampling; (Range 2-24 hrs.).

[b]% injected dose C^{14} in <u>total cecal</u> contents.

[c]Calculated as: (GF-CONV)/(CONV)X100.

This again shows the greater incorporation of C^{14} after l-tryptophane by the germfree rats, especially in the rapidly metabolizing tissues, and the lower incorporation by these same animals of d-tryptophane. "Spillage" of C^{14} into cecal contents is always much greater in the germfree group.

Plasma Clearance Experiment - Figure 5 shows the results of tryptophane assays in the plasma after 25 mg l-tryptophane were infused i.v. in 1% $NaHCO_3$ into a jugular vein. The germfree consistently lagged the conventional group.

Figure 6 shows a similar experiment done with the d-isomer, and the results are the same, except that by 45 minutes post-infusion, the tryptophane levels are only about one-half as great as with the l-isomer.

It thus appears that despite the more efficient metabolism of traces of l-tryptophane by the germfree rat, this animal is less efficient in clearing either l- or d-tryptophane when administered in large loads.

Figure 7 shows rises in plasma urea nitrogen after the loads of tryptophane outlined above. As could be expected, the germfree lags consistently; more surprising is the fact that the d-isomer gives rise to more urea than the l-isomer.

In summary, the germfree rat converts C^{14} l-tryptophane to $C^{14}O_2$ more rapidly than the conventional rat, and incorporates C^{14} into tissue protein, particularly that of the liver and small intestine, more rapidly than the conventional rat. The conventional expires more $C^{14}O_2$ after C^{14} d-tryptophane and incorporates C^{14} more efficiently than the germfree rat into liver and small intestinal protein. The germfree rat expires less $C^{14}O_2$ from the C^{14} d-isomer than from the l-, and incorporates it less efficiently into liver and small intestinal protein; the conventional rat, on the other hand, expires more $C^{14}O_2$ from the C^{14} d-isomer than from the l-, and converts it more efficiently into liver and small intestinal protein. However, both the germfree and conventional incorporates C^{14} from the d-isomer more efficiently than the l- into the large intestine and cecum, and into the cecal content. In either case, more C^{14} appears in the cecal content of the germfree rat. Both d- and l-tryptophane are cleared faster from the plasma of the conventional rat and give rise to more urea; the clearance of d-tryptophane from the plasma of either group is more complete than the l-, and also gives rise to more urea.

Metabolic End-Products of l-Tryptophane in the Urine of Germfree and Conventional Rats - We have planned to fractionate urine into various metabolites of tryptophane (kynurenine, kynurenic acid, xanthurenic acid, anthranilic acid, indoleacetic acid, etc.) in order to determine whether the germfree converts loads of l-tryptophane into these metabolites at different rates from conventional rats. In this way, we had hoped (see above) to be able to trace administered l- and d-tryptophane through expiration of part of their carbon skeleton into expiratory CO_2, appearance of its $-NH_2$ as urea, incorporation of part of the carbon into protein, and finally to determine what part appeared as metabolic end products. For this final project, we required a special diet from which

Figure 1

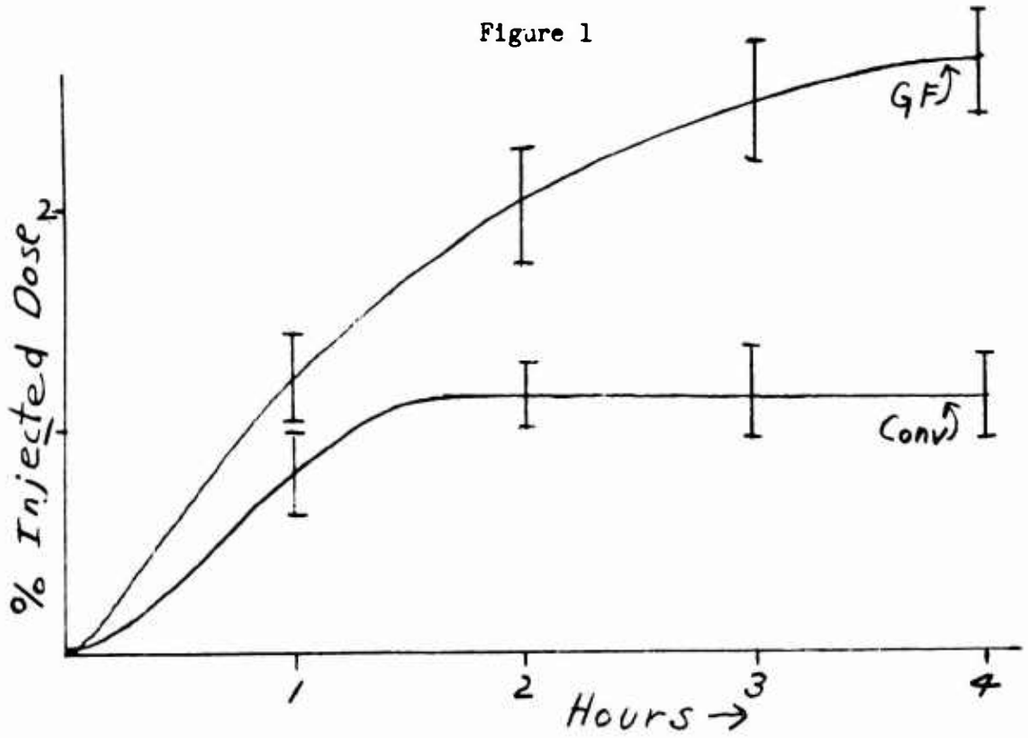


Figure 2

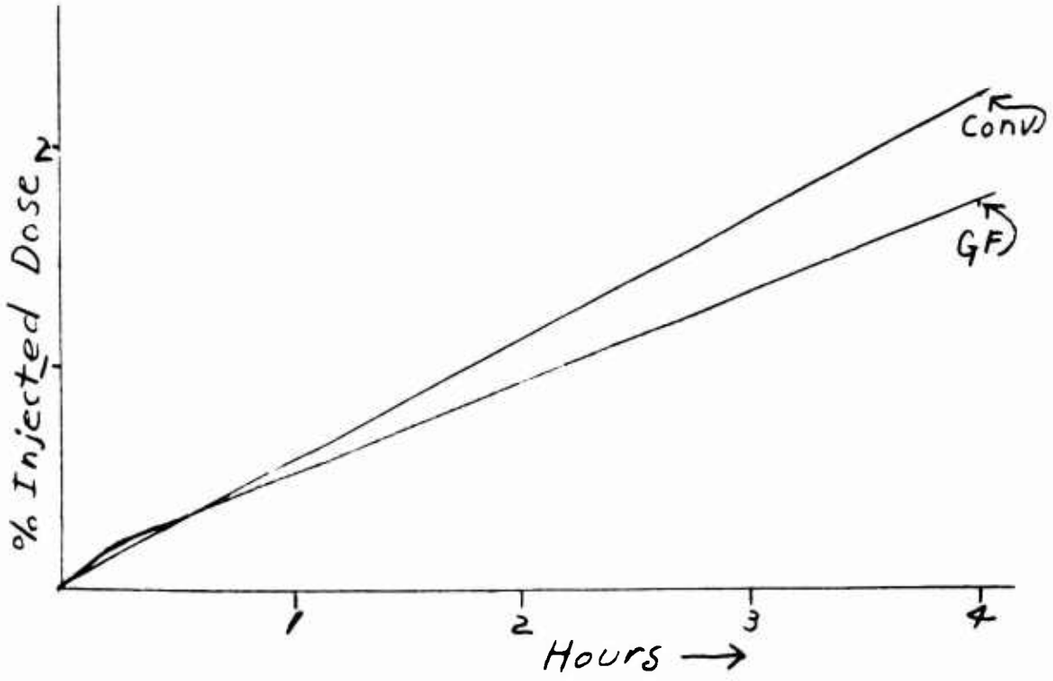


Figure 3

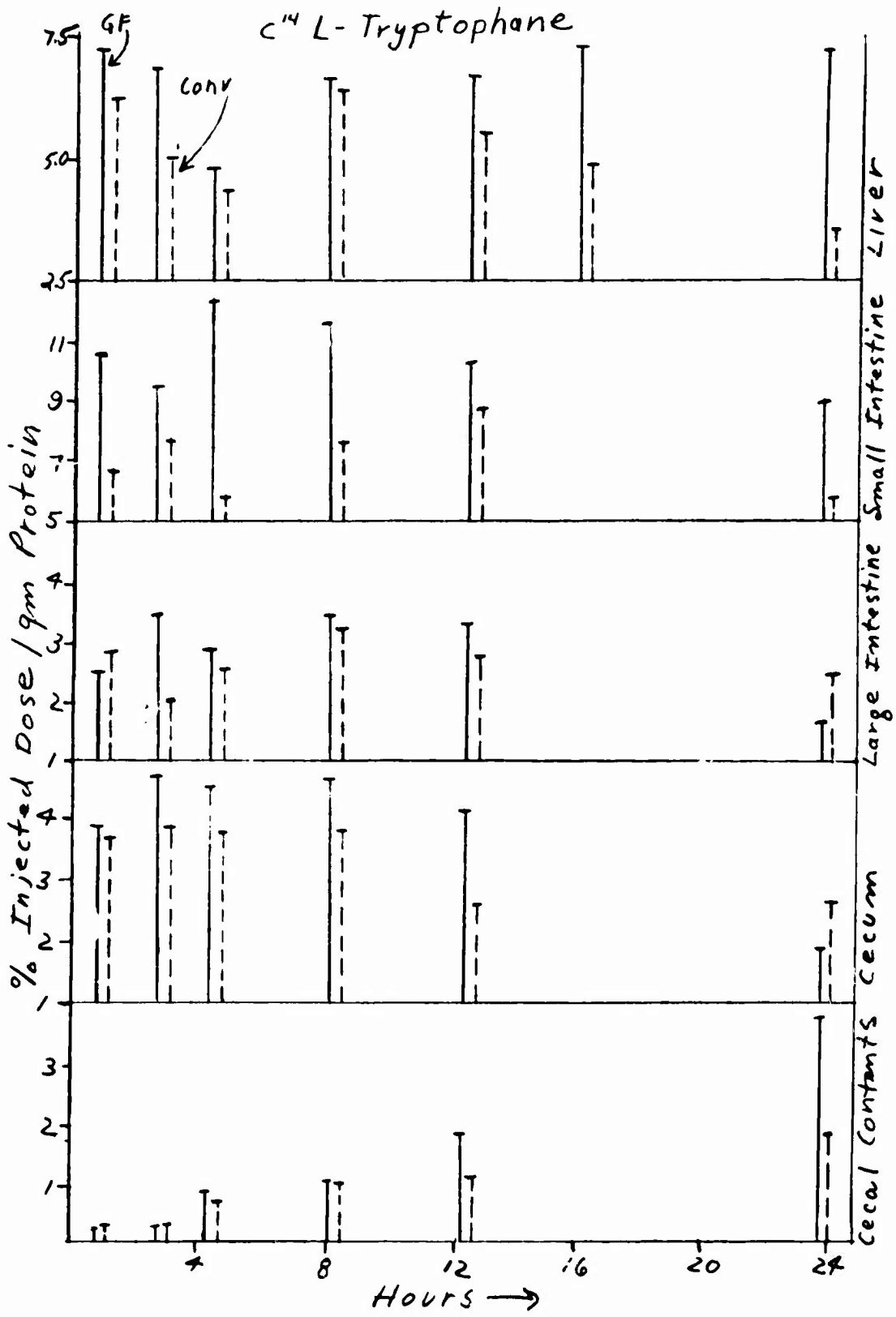

C14 L-Tryptophane
GF
Conv
% Injected Dose / gm Protein
Liver
Small Intestine
Large Intestine
Cecum
Cecal Contents
Hours →

Figure 4

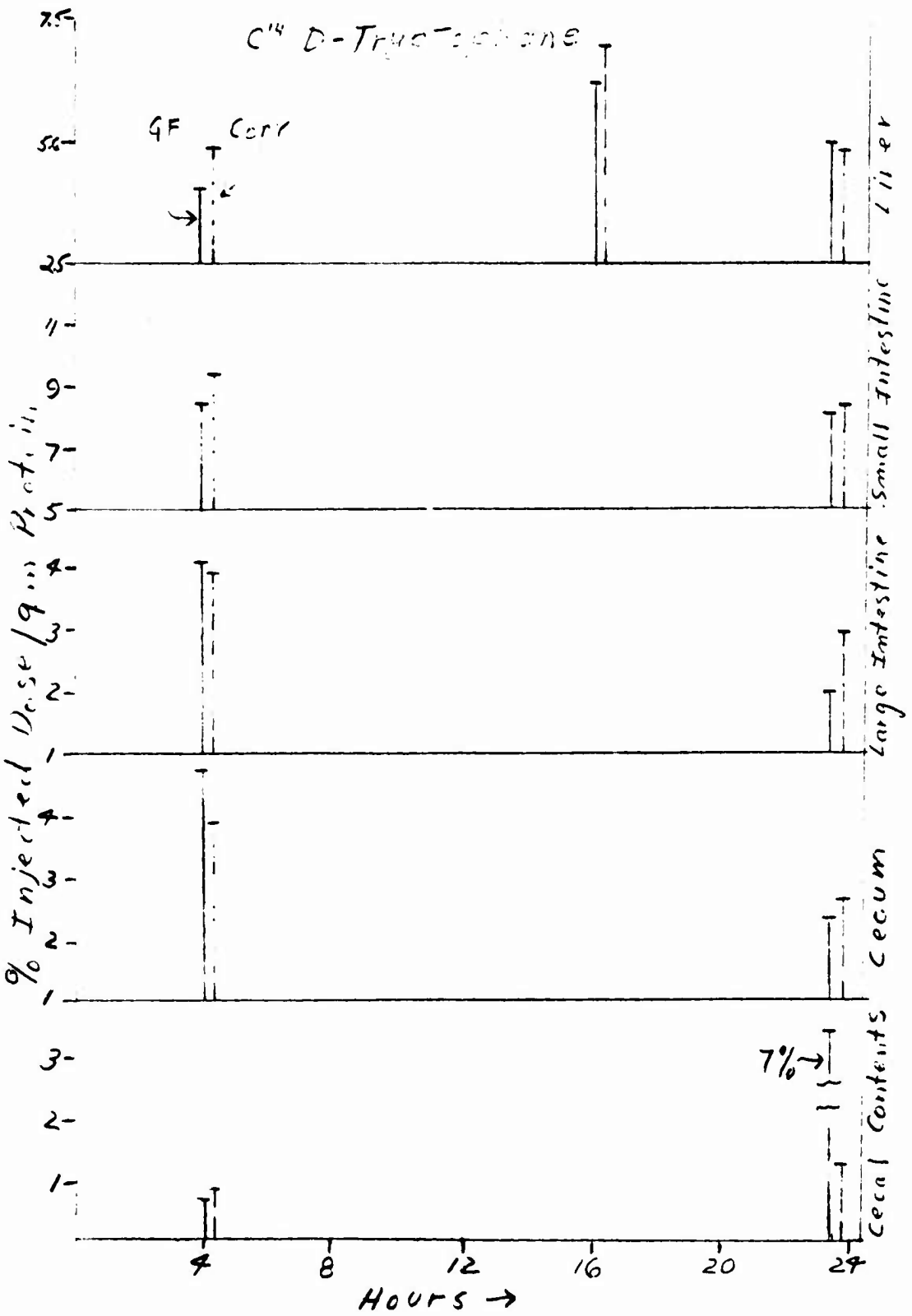

C14 D-Tryptophane
GF Corr
% Injected Dose/gm tissue
Small Intestine
Large Intestine
Cecum
Cecal Contents
7%
Hours →

Figure 5

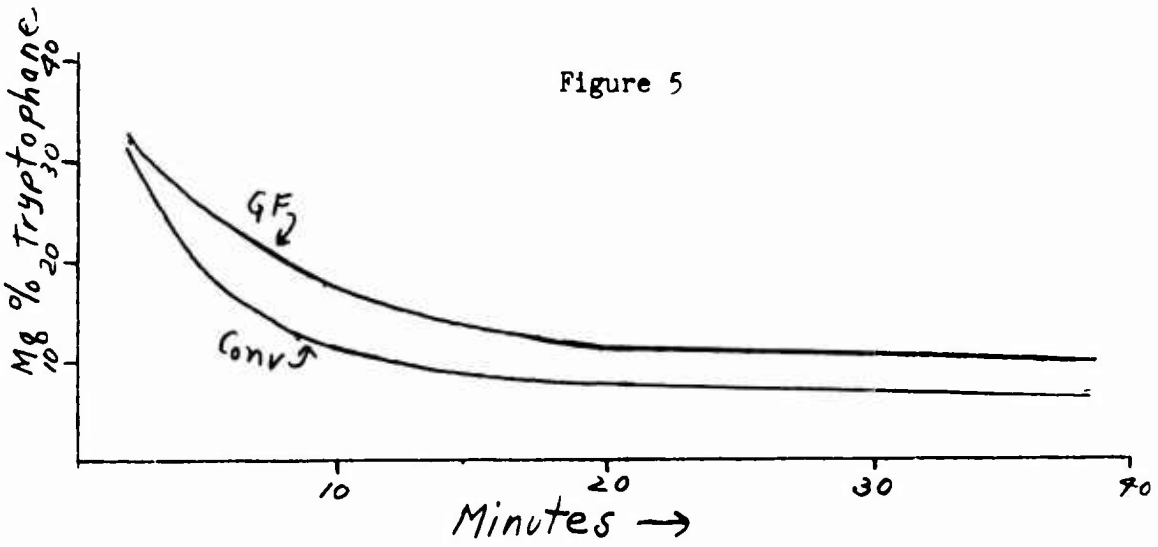


Figure 6

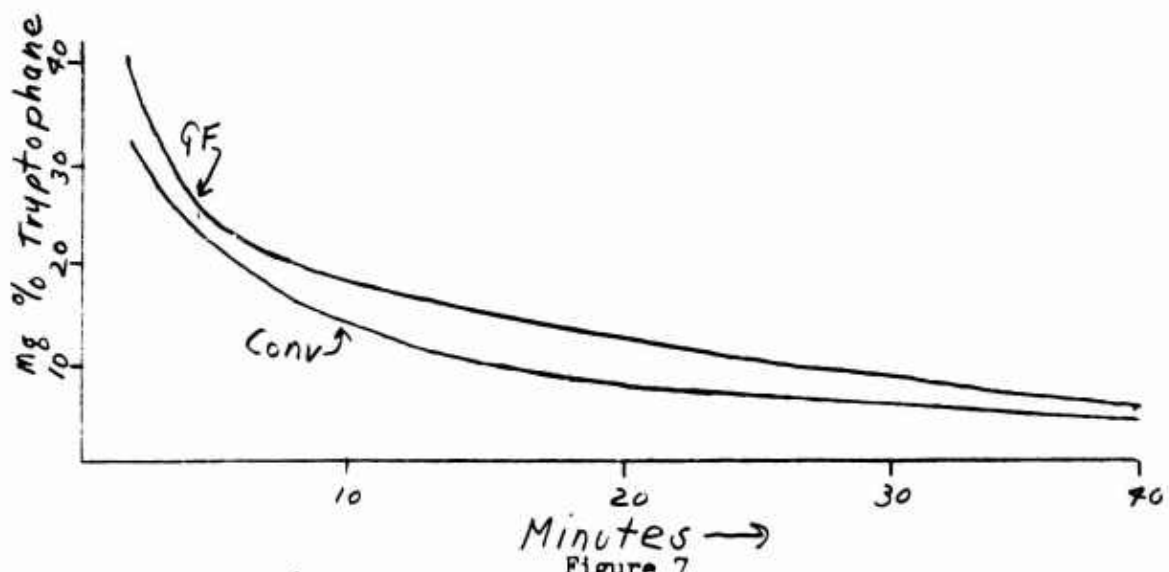
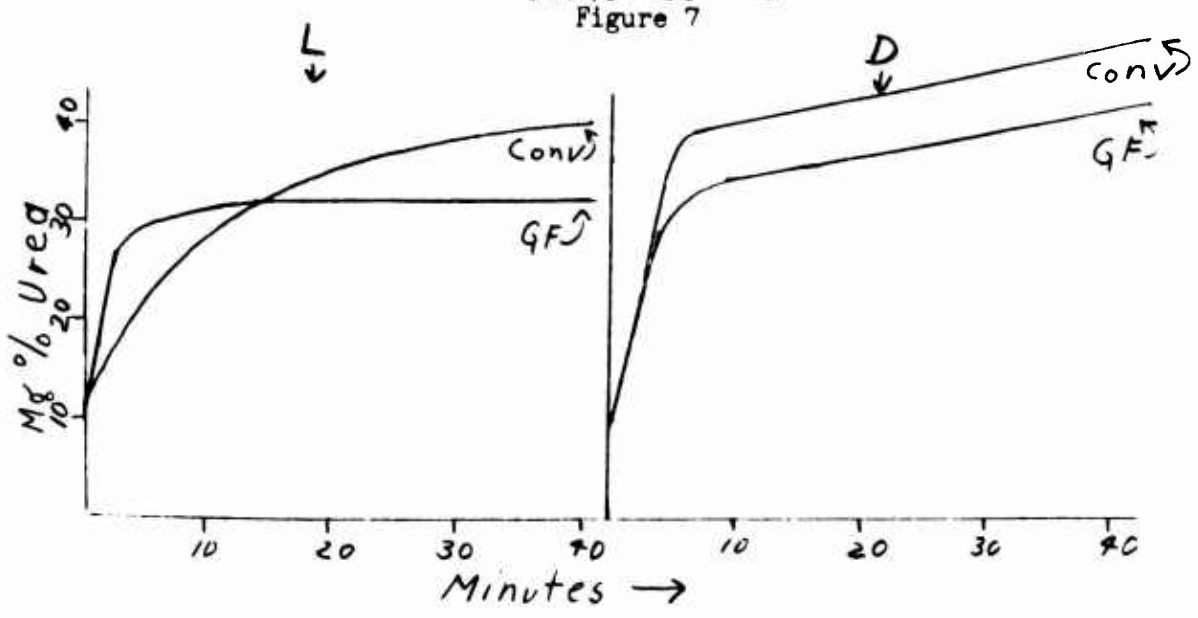


Figure 7



tryptophane is excluded so that we might be able to add either the l- or d-isomer. Such a diet in liquid form is available (#116); this is a wholly synthetic mixture from which protein is excluded, the amino acids being syntnetic. However, previous experience in this laboratory has shown that the germfree rat will not eat this diet, even though the conventional will. We obtained a commercial diet which was a slight modification of #116 and designed a study to test its effectiveness. A new type of isolator was designed and built to allow full metabolic studies in the germfree environment (see elsewhere in report); four germfree and four conventionalized rats were used. The results were as before -- the germfree rats refused the diet, lost weight, and eventually three of them died.

Since metabolic studies of the type outlined above are crucial to us, we are continuing our search for an acceptable diet.

Endocrine Glands and Hormones

Effects of Adrenocortical Steroid Treatment in Aspergillus versicolor Monocontaminated Mice - Sidransky and Friedman (Am. J. Path. 35:169, 1959) have reported that mice develop highly fatal pulmonary aspergillosis (up to 88% mortality) following exposure to spores of the saprophytic fungus Aspergillus flavus when cortisone treatment (5 mg aqueous suspension of cortisone acetate, s.c.) is included. The non-cortisone-treated mice show no mortality. Thus, when an isolator of 9-week-old germfree mice became accidently contaminated with Aspergillus versicolor with no deaths occurring afterwards, the animals were divided into equal groups (27 mice in each) with one group receiving saline injections and the other hydrocortisone sodium succinate (initial dose 5 mg, s.c., followed by 2.5 mg daily for upwards to 4 days, s.c.). Approximately 5 animals from each group were sacrificed daily for 5 days following the initial dose. Portions of the lungs and spleen from each animal were ground under aseptic conditions in sterile physiological saline solution to provide a 10% w/v suspension. The remaining portions of these and of other organs were preserved for histopathological studies.

Tissue suspensions were diluted serially with sterile saline to 10^4. An aliquot of 0.1 ml of each of the 10-fold dilutions, as well as the original suspension, was pipetted individually into tubes of 10-15 ml of sterile melted Sabouraud dextrose agar (1.5 - 1.7% agar) and plated. After 96 to 120 hours' incubation at room temperature, the colonies were counted. The colony counts could then be converted to organisms per gram of tissue.

Of the 14 saline-treated mice which were tested only one plate of each of two mouse spleens showed one colony of Aspergillus versicolor. None of the steroid-treated mouse spleens yielded positive readings. None of the lung tissue suspensions yielded any Aspergillus colonies.

Histopathology After Steroid Administration of Aspergillus versicolor Monocontaminated Mice - Under the conditions of this experiment, hydrocortisone treatment resulted in no histologic evidence of fungal cells in any tissues examined except the alimentary canal. The fungal cells were found in the lumen of the intestine, but with no evidence of budding or mycelial structures. Moreover, neither in the saline- nor steroid-treated mice did the presence of Aspergillus alter the histologic or gross germfree character of the bowel. No infections, gastric ulceration or other complications were in evidence after this vigorous steroid regimen. Lymphocyte depletion was marked in lymph nodes, the spleen, and, particularly, in the cortex of the thymus; similar thymic depletion of lymphocytes is seen in mice 2 to 5 days post-irradiation. The thymus weights of the steroid-treated mice were one-third the values of the saline-treated controls. Adrenal weights did not increase. Histologic studies of other tissues are in progress.

In summary, the persistence of the "germfree" pattern in all organs of the steroid-treated Aspergillus-monocontaminated mice suggests that this treatment did not render the fungus pathogenic, and that the lymphatic tissue changes would probably have been the same in steroid-treated germfree mice. The alterations in the thymus, spleen and lymph nodes would, therefore, appear to be due to the steroid administration per se. The present observations are similar to those reported for steroid-treated bacteria-laden animals except for the reportedly high incidence of infection in these.

As hydrocortisone administration reduces lymphatic tissue mass, further investigation of the comparative histologic and biochemical responses to this treatment exhibited by germfree, defined-flora and conventionalized animals should enable separation of the effects of altered host defenses and infection from the overall metabolic effects of this treatment.

Alloxan Diabetes - Experiments have been conducted in this laboratory to establish a diabetic state in ICR mice via injection of alloxan monohydrate (Eastman Chem. Co.). Pilot studies on germfree, defined-flora, and conventionalized male and female mice were performed to determine the possible role of the indigenous flora with hypoinsulinism. Several problems were encountered during the course of these experiments: blood collection from small animals; blood glucose analyses using small volumes of blood; dose regimen; and sterilization of the alloxan monohydrate for use within our germfree animal-holding isolators.

Perusal of published literature uncovered a technique for taking small, repeated blood samples from the retro-orbital venous plexus of mice with a blood-collecting capillary tube. An ultramicro Glucostat method for analyzing blood glucose has been tested and proven satisfactory. Previous studies involving open colony ICR mice have suggested a dose of 100-250 mg/Kg of 2.5% alloxan monohydrate to be diabetogenic when administered subcutaneously. It was found through spectrophotometric (Beckman DK 1A) investigation that when alloxan was diluted to 2.5% in unbuffered water or unbuffered isotonic saline (pH 5.5 - 6.0) the absorption

maximum was lost within 2-3 minutes. However, when the alloxan was diluted to 2.5% in acetate-buffered saline, at a pH of 2.5 - 3.0, the absorption maximum was stable at room temperature for 18 hours.

Monocontaminated ICR Mice vs. Conventionalized ICR Mice - A pre-weighed quantity of alloxan monohydrate was placed in a vaccine bottle and autoclaved by our standard procedure. The acetate-buffered saline (pH 3.0) was likewise autoclaved and entered into the isolator. The mice were weighed and the dosage of alloxan computed on an individual animal basis. Just prior to use, the alloxan was dissolved in a pre-determined volume of buffered saline to bring the concentration to 2.5%, making allowances for the monohydrate portion of the chemical. Twenty-seven Rhodotorula monocontaminated ICR mice and 30 conventionalized ICR mice of either sex were given a subcutaneous injection of either 2.5% alloxan (diluted with acetate-buffered saline to a pH of 3.0) or a control injection of acetate-buffered saline alone (pH 3.0). All mice were allowed L-356 diet and water ad libitum for 6 days after injection. At this time, the mice were denied both food and water for 24 hours. At the end of the 24-hour fast (7 days post-injection), the mice were bled. The blood sample was immediately processed for blood glucose using the ultramicro Glucostat technique. After being bled, the mice were killed by CO_2 inhalation, grossly examined and preserved for histopathologic examination. The following results were obtained:

Microbial Status	Treatment, s.c.	No. Mice	Blood Glucose, mg% Average	Range
Monocontaminated	2.5% Alloxan*	5	198	91-262
	Acetate-buffered saline	10	129	94-185
Conventionalized	2.5% Alloxan*	6	187	120-251
	Acetate-buffered saline	12	109	93-128

*250 mg/Kg B.W.

The fasting blood glucose level of the alloxan-treated mice, whether monocontaminated or conventionalized, was higher than that of their control-injected counterparts. Our dose of 250 mg/Kg of 2.5% alloxan, however, resulted in a mortality of 62-69%, and is apparently too high.

Another similar experiment involving a small number of germfree and conventionalized ICR mice likewise resulted in elevated blood glucose values for alloxan-treated mice, regardless of bacterial status. Again, over 50% of these mice died before being bled 7 days post-injection.

Histopathology - Alloxan treatment caused varying degrees of renal tubular damage (mainly the proximal convoluted tubules) in all mice, regardless of microbial status; one conventionalized mouse gave evidence of acute pyelonephritis. The kidneys of the control-injected mice were generally not remarkable. The pancreatic islets of most of the alloxan-treated mice showed damage ranging from beta cell destruction alone to complete islet necrosis. The pancreas of control-injected mice of all groups was not remarkable. Alloxan appears to be equally effective in germfree, monocontaminated and conventionalized mice insofar as islet cell destruction is concerned.

In summary, a method has been established for introduction of sterile alloxan monohydrate into isolators and for preparing a stable solution of the same for injection. A fasting hyperglycemia and histologic evidence of beta cell damage 7 days after alloxan treatment indicate that a diabetic state resulted in germfree, yeast-monocontaminated and conventionalized mice. Optimal dosage of alloxan remains to be determined for ICR mice.

Lymphatic System and Immunopathology

Lymphatic Tissue Response to Bacterial and Non-Bacterial Antigen - As previously reported, macrophage digestion of killed E. coli and ensuing antibody formation are faster in conventional than in germfree mice. Sixty germfree and 59 conventional mice now received an organism foreign to the conventional animals' flora (killed Serratia marcescens) into one forefoot and horse ferritin into the other, and were sacrificed from 2 hours to 14 days after inoculation. Axillary nodes and spleens were weighed and studied histologically, autoradiographically (H^3-thymidine), and immunocytochemically for the fate of antigens and immune response morphology. Antibodies were titrated in each animal. Germfree mice had a delayed but vigorous and more prolonged reaction. While no longer demonstrable in conventional nodes by 4-7 days after injection, germfree lymph nodes and macrophages retained Serratia and ferritin throughout the experiment. Bacteriologic sterility and immunologic inexperience thus result in slower intracellular preparation of antigen and delayed but strongly sustained antibody formation regardless of type of antigen. Experience with a bacterial flora quickens but may lessen immune response.

Lymphatic Tissue Response to Liver Damage Induced by a Butter Yellow Derivative - This experiment is designed to determine whether the hypergammaglobulinemia in man and animals with chronic liver disease is related to a direct or adjuvant effect of bacterial flora or to a nonspecific stimulation of immunologically competent cells, autoimmune processes or adjuvant-like effect of tissue breakdown products.

The following model was chosen after several attempts to design a suitable experimental model. A butter yellow derivative, 3-methyl-4-dimethyl aminoazobenzene (3 DAB), a hepatotoxin and hepatocarcinogen, is injected twice weekly (0.5 ml i.p. of a 1.5g/100 ml sesame oil solution) into germfree and conventional rats while control animals received only the diluent sesame oil (0.5 ml i.p.).

Blood was drawn before the first injection and then 21 and 42 days later when the animals were sacrificed. Two separate experiments have been performed, each involving two lots of 20 animals, half of which were germfree and the other half conventionalized. The organs being studied are liver, spleen, perihepatic and axillary nodes and the thymus. Tritiated thymidine was injected one hour before the animals were sacrificed, for radioautographic studies.

The livers showed the expected mild focal liver cell necrosis in the 3 DAB group. Only the first half of the gamma globulin determinations has been done so far. In this first group of 20 animals, gamma globulin titers rose in the germfree group at 21 and at 42 days while it remained at the base line in germfree controls. In the conventionalized rats, the initial globulin levels were higher and both the butter yellow group and control group rose equally at 21 days but the butter yellow group continued to rise while the controls stabilized at the 21-day level. The total protein did not change in any of the groups. Other study phases have yet to be completed. Disclosure of the role of the bacterial flora, if any, in these phenomena may have therapeutic implications. (See Progress Reports on Research Contracts, #DA-MD-49-193-64-G129 to the Mt. Sinai Hosp., N.Y., Dr. Hans Popper; and #DA-49-193-MD-2541, to the Georgetown Univ., Wash. D.C., Dr. Heinz Bauer.)

Tissue Transplantation: Skin Grafting

A technique for reproducibly grafting full-thickness skin was adapted from the method of Gross, Padnos, and Gottfried (Plast. & Reconst. Surg. 25:421, 1960) for use in germfree isolators. The technique used is as follows: Two days prior to grafting, the mice are depilated with Nair. The mice are anesthetized in pairs with halothane (see elsewhere in report). The back is sprayed with a colorless adhesive (Vihesive) and this is allowed to dry. An inch wide strip of adhesive tape (Micropore) is placed transversely on the back. The anterior edges of the graft sites are marked with dye. Two 1/2-inch diameter disks of skin backed with tape are punched out against a sheet of siliconized paper with a Waldemar punch. The disks are replaced on the same or another mouse in the same anteroposterior axis as cut. Each disk is held in place with an 1/8 by 1 inch strip of Blenderm. The area is covered with an inch wide strip of Blenderm rendered non-adhesive over the grafts by another strip of Blenderm placed so that the adhesive surfaces are together. This acts as a splint and a protective covering. A plastic collar, approximately 5-1/2 cm in diameter with a 1-1/2 cm diameter neck hole in it is placed around the mouse's neck to prevent it from reaching back and harming the grafts. The mice are individually housed until sacrifice.

With this technique, the course of healing of full-thickness skin grafts, in which both bed and graft are precisely duplicated, has been studied in germfree and conventionalized mice. Two homografts, two autografts, or one of each could be observed in each mouse. The animals were sacrificed and studied 4, 5, 6, 8, 9, 11, 14 and 28 days after grafting. Gross observations were made at the time of sacrifice, and

serum was obtained by heart puncture for electrophoresis. The animals were fixed in 10% buffered formalin for histologic evaluation.

Six separate experiments with 450 grafts on 225 mice were performed during the past year. Complete evaluation of all this material has not been completed, but certain definite conclusions have been reached about the healing of autografts.

The technique was feasible for large numbers of grafts under the restrictions imposed by germfree isolators. The grafts were revascularized from the subdermal vessels of the surrounding skin, not from beneath. Revascularization occurred before 4 days. Grafts not revascularized by this time were not viable. A moderate amount of epidermal and hair follicle necrosis occurred during this time. Thereafter, reepithelialization occurred and hair grew in the graft. The source of the new epithelium was not determined. A large number of capillaries was evident in the graft by gross examination with transillumination at 6 days. Far fewer blood-filled capillaries were evident at 8 days, and by 11 days, the vascularity of the graft was essentially that of the surrounding skin. Inflammatory cells and edema were seen microscopically in the grafts during the 4 to 10-day period. By 14 days, the inflammation in the graft had subsided and healing appeared complete histologically. Little contraction of the grafts was observed. The regional (axillary) lymph nodes enlarged and showed evidence of intense immunologic activity after autografting in both germfree and conventionalized mice; the maximum response was observed at 9 days. Autografts healed better in the germfree mice than in the conventionalized because of some amount of infection in about 15% of the latter. It is of interest that none of the bacteria thought to be of great importance clinically in the destruction of skin grafts, alpha hemolytic Streptococcus, Staphylococcus aureus, or Pseudomonas, were present in these animals. A limited number of serum protein separations were carried out by agar gel electrophoresis. More work is necessary before any conclusions in this regard can be made.

Homografts were transferred between animals of the same sex within ICR and CD1 (Charles River designation) strains, and between Balb/c and ICR strains. Rejection of these grafts in the classical sense was not observed, although the percentage of takes of homografts was less than that of autografts in the ICR mice. The same sequence of events was observed grossly and histologically in the homografts as in the autografts with the exception that more of an inflammatory response was observed in the former. This response, however, was of the polymorphonuclear leukocyte variety and not of lymphocytic or plasma cell infiltration. Further studies are in progress to define the nature of this very interesting phenomenon.

Summary and Conclusions.

Using germfree (GF) biotechnology, multidisciplinary comparative studies were conducted on GF, defined-flora ex-germfree, and conventional(ized) animals in order to isolate the bacterial from the non-bacterial (host) factors and delineate their beneficial or detrimental effects on the body economy in health or after injury, shock and disease states induced by various agents and stressors, and to learn to manipulate the indigenous and environmental microorganisms or their effects to the best advantage of the host. We have found that the microbial status of the host significantly influences morphology, GI physiology, protein metabolism, and response to chlortetracycline, antigens, bowel ischemia, uremia and wound healing. Fatal abacterial peritonitis occurs in GF rats from a leaking suture line of the cecum. Hyperoncotic 10% Low Molecular Weight Dextran is not an effective blood substitute for the monkey after hemorrhagic shock. Irrespective of microbial status, saline therapy improves survival time after bowel or limb ischemia or burns.

Studies are continuing on: antibacterial vs. non-antibacterial (toxic) actions of antibiotics; alterations of GI tract by microbial flora; immunological response of lymphatic system after antigens and liver damage; hormonal effects (adrenal steroids, alloxan diabetes); amino acid and protein metabolism and enzyme induction; skin grafting and wound healing; shock induced by limb or bowel ischemia and burns; anuric states (biochemistry and survival with uremia); derivation, maintenance and study of GF primates (monkey).

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PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 05
Microbiology

RESEARCH AND TECHNOLOGY RESUME

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C. IN-HOUSE	NA	PRIOR FY 65	5	84
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19. GOV'T LAB/INSTALLATION/ACTIVITY
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22. COORDINATION: NA

23. KEYWORDS: Molecular biology; biochemistry; biophysics; microbiology; antimalarials; chloroquine; quinacrine; antibiotics; chloramphenicol; streptomycin; tetracyclines

24. (U) Tech Objective - Elucidation of modes of action of antimalarial and antibiotic agents at the molecular level; specifically, of mechanisms by which such agents interfere originally with biological replication of macromolecules and consequently with growth and replication of microorganisms. Such principles of antimicrobial action when recognized, provide points of departure into premeditated design of superior drugs; they also explain observations on sensitivity or resistance to chemotherapeutics.

25. (U) Approach - Experimental studies (employing methods of biochemistry, biophysics, microbiology, or, in summation, molecular biology) on biosynthesis of DNA, RNA, and protein in cells and partly resolved cell-free systems; physical studies on binding of drug molecules to biological macromolecules, for example chloroquine to DNA or chloramphenicol to ribosomes. Theoretical studies on relationships between chemical structure and biological activities. Major scientific problems are encountered in the area of biosynthesis of protein and of DNA, both processes being incompletely resolved into their component reactions.

26. (U) Progress (Jul 64 - Jun 65) - Chloroquine binds to DNA and inhibits the replication of DNA as well as the transcription of RNA from DNA; this is accompanied by massive breakdown of ribosomes and exponential decrease in viability. A generalized prototype structure of antimalarials of the chloroquine-mepacrine type has emerged. Oxytetracycline, chloramphenicol and streptomycin inhibit protein synthesis at the ribosomal level.
For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 05, Microbiology

Work Unit 030, Modes of action of antimicrobial agents

Investigators.
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Description.

Elucidation of the mechanisms of action of antimalarials and antibiotics at the molecular level with a view to explaining antimicrobial action and/or resistance thereto and providing guidelines to logically premeditated chemotherapy research.

Progress.

Mode of Action of Chloroquine

The preceding ANNUAL PROGRESS REPORT proposed the hypothesis that chloroquine exerts its antimicrobial action primarily through reacting with DNA. This hypothesis is now supported by several lines of evidence.

Biochemical knowledge on the mode of action of chloroquine had advanced slowly because of the unavailability of suitable test organisms. Schellenberg and Coatney (Biochem. Pharmacol. 6: 143, 1961) reported that chloroquine inhibited the incorporation of radioactive phosphate into the nucleic acids of Plasmodium berghei and Plasmodium gallinaceum; from this we inferred that the drug may be an inhibitor of nucleic acid biosynthesis. Despite reports that chloroquine did not have any effect on the growth of bacteria, we carried out a series of screening experiments and found a strain of Bacillus megaterium which is susceptible to chloroquine at concentrations of the order of 10^{-4} to 10^{-3} M. The following work was carried out with this organism.

Turbidimetric measurements showed that B. megaterium growing in a modified Sauton medium, had a duplication time of approximately one hour. When chloroquine was added to such a culture, growth was inhibited immediately and cell mass increased only during the first 20 minutes by approximately 10 per cent. The inhibition of growth by chloroquine was accompanied by a rapid decline in the number of colony-forming organisms. When samples were removed from chloroquine-treated cultures at time intervals and subjected to serial dilution plate counting, it was found that the number of viable bacteria decreased by three orders of magnitude ("logs") during one duplication time. Hence, chloroquine acted as a strong bactericidal agent.

Biochemical analyses on aliquots of bacteria taken at time intervals from chloroquine-exposed cultures showed that chloroquine inhibited completely the biosynthesis of DNA in B. megaterium. After eliminating the growth medium and the contents of the metabolic "pool" by cold perchloric acid extraction, the nucleic acids of the organisms were extracted with hot perchloric acid and DNA was determined by a diphenylamine method. During an experimental period of one duplication time, the amounts of DNA per aliquot remained constant, i.e., DNA was neither synthesized nor degraded and eliminated by chloroquine-exposed bacteria.

Analysis for total RNA in the nucleic acid extracts by an orcinol-$FeCl_3$ method revealed that global RNA not only failed to increase in quantity, following the addition of chloroquine, but that the test organisms actually lost 12 per cent of their total RNA during one duplication time. When the bacterial global RNA had been pre-labelled with radioactive uracil prior to exposure of the culture to chloroquine in a non-radioactive medium, the organisms lost approximately 10 per cent of their radioactivity progressively into the experimental medium, confirming independently the occurrence and extent of RNA-breakdown owing to the action of chloroquine.

In order to gain deeper insight into the dynamic events produced by chloroquine in RNA metabolism, the total nucleic acids of B. megaterium were extracted with a phenol method following mechanical disruption of the cells by sudden release from hydrostatic pressure. After removal of the phenol, the nucleic acids were precipitated with ethyl alcohol, redissolved in 0.3 M. sodium chloride and subjected to chromatography on a methylated albumin-kieselguhr ("MAK") column by elution with a linear gradient of sodium chloride between 0.5 and 1.0 M. The transfer-RNA (4 s) fraction remained unaffected by exposure of the bacteria to chloroquine. There was more UV-absorbing material preceding DNA and eluting in the DNA region in samples from chloroquine-treated bacteria than in samples from normal cultures. The amounts of 16 s and 23 s ribosomal RNAs, however, were dramatically reduced in chloroquine-exposed bacteria to the extent that the 16 s component largely disappeared. When radioactive uracil had been present in the medium of chloroquine exposed B. megaterium, significant radioactivity was detected in the 4 s peak but little activity was found in the 16 - 23 s region.

It is concluded that one of the consequences of the action of chloroquine is a marked breakdown of ribosomal RNA. A small portion of this material is degraded to an extent that the fragments are excreted by the test organism (approximately 14 per cent of the ribosomal RNA) but most of it remains in the cells in form of dissimilation products of no preferred molecular size, some of it apparently larger than 23 s. This massive dissimilation of ribosomal RNA suggested to us the possibility of a concomitant dissimilation of the ribosomal particles.

When cell-free preparations of ribosomes were suspended in solutions of chloroquine and incubated for 60 minutes, subsequent sucrose gradient (5 to

25 per cent) centrifugal analysis showed that the distribution of these particles in the gradient did not differ significantly from that of bacterial ribosomes that had not been treated in vitro with chloroquine. In contrast, when ribosomes from whole bacteria in cultures exposed to chloroquine were prepared and anlyzed by passage through a molecular sieve (Sephadex G-100) it was found that the amount of ribosomes per bacterial aliquot declined rapidly as a function of the time of exposure of the bacteria to the drug. After 30 minutes of treatment with chloroquine, cells of B. megaterium had lost 77 per cent of their ribosomes. This loss was most pronounced during the first 20 minutes of chloroquine action and then proceeded at a decreasing rate.

The effects of chloroquine upon B. megaterium were not the result of a generalized metabolic failure such as might result, for example, from an inhibition of cellular respiration. At bactericidal concentrations of the drug which produced the inhibitions of nucleic acid synthesis and the ribosomal degradation described above, chloroquine inhibited the oxidation of glucose in B. megaterium by only 10 per cent. A probit transformation of the response of glucose oxidation to the dosage of chloroquine showed the ED_{50} to be of the order of 2.4×10^{-3} M, and complete inhibition would require, by extrapolation, 3×10^{-2} M or 10 mg./ml.

The growth-inhibitory and bactericidal effects of chloroquine are plausibly the results of an inhibition of DNA and RNA synthesis as well as of the degradation of ribosomal RNA and of ribosomes. It was thought that these effects are caused by the formation of a complex of chloroquine with DNA.

A number of antimicrobial substances form complexes with DNA and produce biological effects by inhibiting reactions in which DNA participates; this has been demonstrated for mitomycin C, actinomycin D, proflavine, daunomycin, cinerubin, chromomycin A_3, echinomycin, miracil D, and ethidium bromide. In the preceding Annual Progress Report, preliminary results were communicated which indicated that chloroquine also formed a complex with DNA.

A most general and direct method of demonstrating the existence of a complex of DNA with a light-absorbing substance is to observe the sedimentation of such a complex in the analytical ultracentrifuge. We have followed the sedimentation of calf thymus DNA in the absence as well as in the presence of chloroquine by photographing the schlieren pattern projected by light from a mercury lamp passing through a Corning filter #5840; this light source consists predominantly of the mercury lines at 365/66 mμ. Since chloroquine is optically dense at this wavelength (A^{365} of the DNA-chloroquine mixture centrifuged was 0.50 for the centrifuge cell with an optical path of 1.2 cm) it was possible to determine that the drug sedimented with the DNA. This finding confirms directly the conclusions from less direct experiments that chloroquine forms complexes with DNA.

Difference spectrophotometry showed further that double-stranded, native DNA produced very marked changes in the absorption spectrum of chloroquine while only minor changes were produced by DNA that had been heat-denatured in the presence of 0.33 M formaldehyde and was, therefore, unable to reconstitute into a double helix; equally minor changes were produced by DNA at 98° C, i.e., at a temperature at which DNA is single-stranded. These slight spectral changes produced by single-stranded DNAs resemble those produced by heparin, a polysaccharide which shares with nucleic acids the property of possessing one mineral anionic group per molecular weight equivalent of the order of 3×10^2 and is useful as a polyanionic control substance in studies on specific complex formation of antimicrobial substances with DNA.

The strong influence of double-stranded DNA upon the spectrum of chloroquine suggested that the drug is bound to both complimentary strands of DNA. This was expected to have an effect upon the stability of the helix. Indeed, the elevation of the median thermal dissociation temperature, T_m, of DNA by chloroquine which was reported in the preceding Annual Progress Report argues in favor of this idea. These studies have been extended to include a DNA-like polymer consisting of a double-helical structure of strands in which deoxyadenylic acid and thymidylic acid alternate monotonously; the thermal dissociation of this double-stranded poly dAT was influenced by chloroquine to the same extent as that of native complete DNA. This last finding suggests that neither deoxyguanylic acid nor deoxycytidylic acid, the two DNA components missing in poly dAT, are essential in the type of interaction between the polymers and chloroquine that produces increases in T_m.

Kurnick and Radcliffe (J. Lab. Clin. Med. 60: 669, 1962) have observed an enhancement of the viscosity of DNA solutions by chloroquine; this suggests that the drug complexes with DNA in a manner which resembles the interaction with mepacrine and other acridines, viz., through an insertion or "intercalation" of the heterocyclic ring system between base pairs in DNA approximately perpendicular to the long axis of the double helix. Such an effect should lead to a seemingly paradoxical decrease in the sedimentation coefficient of DNA; preliminary findings in this laboratory have shown, indeed, that the sedimentation coefficient of DNA was decreased by chloroquine from 21 to 17, a very substantial decrease.

Intercalation of the 7-chloro-quinoline ring system does not necessarily explain a stabilization of the helix in the sense of an increased resistance to thermal dissociation. The two non-heterocyclic amino groups of chloroquine in its side chain, however, are separated by four carbon atoms: the drug may be considered a substituted 1,4-diaminopentane. Among primary aliphatic diamines of graded chain lengths, diamino-butane and diaminopentane are strongest in elevating T_m of DNA (Mahler and Mehrotra, Biochim. Biophys. Acta 68: 211, 1963) although concentrations of the range of 10^{-3} M are required to produce effects comparable to those of 10^{-5} M chloroquine.

Spermine, on the other hand, which possesses two secondary amino groups separated by four carbon atoms is as active as chloroquine at equivalent molar concentrations in elevating T_m of DNA (Tabor, Biochemistry 1: 496, 1962). We assume that non-primary diaminobutanes stabilize the DNA helix by ionic interaction with phosphoric acid groups across the minor groove of the Watson-Crick-Wilkins DNA model. The bond distances and ionic radii of a secondary diaminobutane add up to approximately 10Å which is the interval between phosphate residues across the minor groove. Chloroquine, according to this hypothesis, intercalates with its quinoline ring between base pairs in DNA and links the two complementary strands by means of its protruding side chain. These assumptions concerning the nature of the chloroquine-DNA complex and the requirements for the structure of chloroquine to provide for an ideal fit to DNA are consonant with information on the relationship between structure and antimalarial activity of chloroquine congeners.

The direct biochemical consequence of the formation of a complex of chloroquine with DNA should be an inhibition of enzymatic reactions in which DNA participates either as a substrate (DNAase), or as an organizer for its own replication (DNA polymerase) or finally as an organizer for the transcription of RNA (RNA polymerase). Kurnick and Radcliffe (loc. cit) have reported that chloroquine inhibited the hydrolysis of DNA by a serum DNAase; more conclusive studies in this laboratory have shown that the hydrolysis of DNA by pancreatic DNAase is, indeed, inhibited by chloroquine.

We have finally shown that the actions of isolated bacterial DNA polymerase and RNA polymerase are inhibited by chloroquine; the DNA polymerase reaction was more sensitive to the drug than the RNA polymerase reaction. Similar enzymological studies have been carried out in other laboratories for several of the DNA-complexing antimicrobial agents cited above and have in all instances succeeded in demonstrating inhibitions of the reactions in which DNA participates as an organizer ("primer").

We conclude that chloroquine by virtue of forming a complex with DNA inhibits the replication of DNA (DNA biosynthesis) as well as the transcription of RNA (RNA biosynthesis) and that these effects are likely candidates for the mode of action of chloroquine at the molecular level. The dramatic breakdown of ribosomes in vivo is probably a secondary effect for which precedents are to be found in the literature in those instances in which DNA synthesis is primarily inhibited (Suzuki and Kilgore, Science 146: 1585, 1964).

Mode of Action of Mepacrine

Preliminary studies on the mode of action of mepacrine were facilitated by the facts that (1) the drug is an effective antibacterial agent, and (2) the formation and structure of the mepacrine-DNA complex has been extensively studied (Kurnick and Radcliffe, loc. cit.; Lerman, Proc. Nat. Acad. Sci. U.S. 49: 94, 1963; J. Cellular Comp. Physiol. 64: Suppl. 1, 1, 1964).

Two common laboratory strains of Escherichia coli were found to be sensitive to 2.5×10^{-4} M mepacrine. At drug concentrations slightly below the minimal growth inhibitory levels, the bacteria formed extremely long filaments to an extent rarely seen under the influence of antimicrobial agents. Fluorescence microscopy revealed that these giant filamentous forms contained mepacrine while the occasional single bacterial cells seen in the micrcscope contained much less of the drug. Electron microscopy of the filaments showed an irregular distribution of somatic matter inside long tubulous cell walls with a rare occasional cross-septum. This visible evidence of impaired binary fission of mepacrine-exposed bacteria suggested strongly that cytokinesis was inhibited as the result of a primary and specific inhibition of DNA replication. Such an effect is produced, for example, when DNA replication is inhibitied by mitomycin C (Shiba et al., Biken's J. 1: 179, 1958).

Chemical analysis of E. coli aliquots from cultures inhibited by mepacrine confirmed readily that DNA replication was strongly inhibited. Complete biochemical studies on mepacrine-exposed E. coli are in progress.

Enzymological studies with isolated DNA polymerase and RNA polymerase of bacterial origin revealed that mepacrine was an inhibitor of both enzymatic reactions and that the DNA polymerase reaction was more strongly influenced by the drug than was the RNA polymerase reaction. Molecule for molecule, mepacrine showed a greater activity than did chloroquine.

Schellenberg and Coatney (loc. cit.) have reported that mepacrine inhibited the incorporation of radioactive phosphate into the nucleic acids of Plasmodium berghei and Plasmodium gallinaceum, and as in the case of chloroquine, we have inferred that the drug was an inhibitor of nucleic acid biosynthesis. Our preliminary studies are in accord with this hypothesis.

In confirmation of early studies (Fischl and Singer, Zschr. f. Hyg. 116: 348, 1934) we have also shown by fluorescence microscopy that malarial parasites, parasitizing erythrocytes (P. berghei in mouse erythrocytes), readily accumulated mepacrine in contrast to the soma of erythrocytes; there was complete parallelism between the incidence of malarial parasites as observed by dark-field microscopy and the incidence of mepacrine fluorescence as observed by fluorescence microscopy in identical microscopic fields. This marked accumulation of the drug in malarial parasites does not only afford a method for rapid parasite counts and/or diagnosis but suggests that the effective concentrations of mepacrine in plasmodia are significantly higher than would be suggested by the plasma levels of mepacrine in patients receiving the drug (approx. 2×10^{-7} M).

Mepacrine has recently gained greatly in interest through observations that it can prevent the emergence of drug resistance in bacteria in vitro as well as in patients receiving antibacterial chemotherapy (Sevag, Arch. Biochem. Biophys. 108: 85, 1964; personal communication October 1964). We are entertaining the hypothesis that this phenomenon, which is of the greatest theoretical

and practical importance, is related to the formation of specific complexes of mepacrine with the genomes (DNA) of drug-sensitive bacteria.

Mode of Action of Streptomycin

Preceding Annual Progress Reports have been concerned with the action of streptomycin as an inhibitor of protein biosynthesis and with the distortion of the normal pattern of RNA biosynthesis in streptomycin-exposed bacteria. Recent communications have indicated, however, that streptomycin and other aminoglycoside antibiotics do not only inhibit protein synthesis strongly but also produce ambiguous polymerizations of certain amino acids in cell-free ribosomal systems supplied with polyuridylic acid (poly U) as one model of messenger RNA (Davies et al., Proc. Nat. Acad Sci. U.S. 51: 883, 1964).

The actual reading of the RNA code in protein synthesis involves individual acts of recognition of codons in messenger RNA vs. anticodons in transfer RNA; this isolated step has become amenable to study by a method (Nirenberg and Leder, Science 145: 1399, 1964) in which complexes of ribosomes, messenger RNA, and radioactive amino acyl-transfer RNA are adsorbed onto nitrocellulose membrance filters (Millipore) while uncombined messenger RNA and amino acyl transfer RNAs pass through such filters.

By employing this method we have shown that streptomycin does cause ambiguities in codeword recognition. As a function of the transfer RNA concentration, the binding of lysyl-transfer RNA or of phenylalanyl-transfer RNA to ribosomes of E. coli complexed with poly A or poly U, respectively, was slightly altered. Polyadenylic acid (poly A) and poly U are the messenger RNAs for the biosynthesis of polylysine or of polyphenylalanine by virtue of the fact that AAA is the codeword for lysine and UUU the codeword for phenylalanine. The inhibitions of these natural codeword recognitions by streptomycin were much less pronounced than are the effects of the antibiotic upon the polymerizations of these amino acids in cell-free ribosomal systems stimulated by poly A or poly U (Van Knippenberg et al., Biochim. Biophys. Acta 80: 526, 1964; Speyer et al., Proc. Natl. Acad. Sci. U.S., 48, 684, 1962; Flaks et al., Biochem. Biophys. Res. Comm. 7: 385, 1962).

In contrast, streptomycin strongly enhanced the poly U-directed binding of isoleucyl transfer-RNA to ribosomes; this binding represents an ambiguity in codeword recognition since the codeword for isoleucine is not UUU but UUA or an anagram of this word. Davies et al., (loc. cit.) have noticed that amino acid incorporation ambiguities produced by streptomycin involved only amino acids whose codons are logically connected. A systematic study of the response of the binding system to the dosage of streptomycin revealed that maximal ambiguous binding of isoleucyl transfer RNA to poly U-informed ribosomes occurred when the number of streptomycin molecules present (4.6×10^{14}) approximated the estimated number of ribosomes (3.8×10^{14}); increases in streptomycin concentrations beyong this equivalence

range did not produce significant increases in ambiguous code word recognition. Streptomycin is known to possess one site of action on the 30 s subunit of ribosomes (Davis, Proc. Natl. Acad. Sci. U.S., 51: 659, 1964; Cox et al., ibid, 703). It is inferred that the interaction of one molecule of the antibiotic with one site on the 30 s ribosomal subunit is sufficient to cause maximal code perturbations.

Our results support the hypothesis of an alteration of the coding properties of ribosome-combined poly U by streptomycin (Davies et al., loc. cit.) although the ambiguous recognition and ribosomal binding of isoleucyl transfer RNA did not occur at the expense of a decreased binding of phenylalanyl transfer RNA. Quite possibly, streptomycin enhances coding ambiguity by activating and altering the reactive properties of codons in poly U that are not regularly engaged in the direction of phenylalanyl transfer-RNA binding. This idea may explain the observation (Pestka et al., Proc. Nat. Acad. Sci. U.S. 53: 639, 1965) that a significant stimulation of coding ambiguity by streptomycin requires that the directing oligo U have a chain length of > 12 U.

A gross misreading of the amino acid code in vivo and a resulting "flooding" of bacterial cells with non-functional proteins have been proposed as causes for the bactericidal effect of streptomycin (Davies et al., loc. cit.). In streptomycin-exposed bacteria any massive synthesis of protein appears to be ruled out by the results of detailed chemical analysis (Hahn et al., Biochim. Biophys. Acta 61: 741, 1962) and all suggestions of some form of streptomycin-induced lethal protein synthesis must take into account the fact that the antibiotic is per se a potent inhibitor of protein synthesis. In our opinion, streptomycin jams the progression of protein synthesis by combining with the 30 s subunit of ribosomes: the induction of coding ambiguities is an interesting corollary of the quantitatively more prominent effect of inhibition of protein biosynthesis.

Mode of Action of Chloramphenicol

The problem of the mechanism of action of chloramphenicol can be reduced to the task of formulating and investigating a working hypothesis that must explain three basic facts: (1) one molecule of chloramphenicol binds per 50 s subunit of ribosomes (Wolfe and Hahn, Biochim. Biophys. Acta 95: 146, 1965); (2) the base composition of messenger RNA modifies most strongly the potency of chloramphenicol to inhibit model protein synthesis in cell-free ribosomal systems (Speyer et al., Cold Spring Harbor Symp. Quant. Biol. 28: 559, 1963; Kucan and Lipmann, J. Biol. Chem. 239: 516, 1964); and (3) chloramphenicol inhibits the isolated process of peptide bond synthesis (Nathans, Proc. Nat. Acad. Sci. U.S. 51: 585, 1964; Traut and Monro, J. Mol. Biol. 10: 63, 1964).

One possible mechanism by which chloramphenicol might bring about these three effects was an inhibition of the codon-anticodon interaction, i.e., an

interference with the reading of the code. The experimental method of Nirenberg and Leder (loc. cit.) was employed to test this hypothesis with the result that the effects of chloramphenicol on the poly U-directed binding of phenylalanyl transfer-RNA or the poly A-directed binding of lysyl transfer-RNA to bacterial ribosomes were not significantly inhibited by the antibiotic (Wolfe and Hahn, Fed. Proc. 24: 217, 1965). Evidently, the act of code word recognition is not the process in protein synthesis that is inhibited by chloramphenicol; similar conclusions have been reached by Suarez and Nathans (Biochem. Biophys. Res. Comm. 18: 743, 1965). The problem still revolves around the central question raised in the preceding Annual Progress Report as to the nature of the structure and function of ribosomal particles as production units in protein synthesis.

Mode of Action of Oxytetracycline

Tetracyclines are inhibitors of protein synthesis and of cell-free model systems of protein synthesis (Rendi and Ochoa, J. Biol. Chem. 273: 3711, 1962; Franklin, Biochem. J. 87: 449, 1963; Laskin and Chan, Biochem. Biophys. Res. Comm. 14: 137, 1964). Unlike chloramphenicol, oxytetracycline was found to produce a considerable inhibition of the binding of lysyl transfer-RNA to poly A-ribosomalcomplexes. Analogous findings have been reported most recently for the effects of tetracycline on the binding of phenylalanyl transfer-RNA to poly U-ribosome complexes (Suarez and Nathans, loc. cit.) and of chlortetracycline upon that same system (Hierowski, Proc. Nat. Acad. Sci. U.S. 53: 594, 1965). Apparently, the tetracyclines interfere with the individual acts of codon recognition in protein synthesis.

The dependency of this inhibition upon the concentration of Mg++ was studied in detail in this laboratory; it was found that the inhibitions produced by a given concentration of oxytetracycline were proportional to the Mg++ concentrations above certain threshold values and that these concentrations thresholds, in turn, were directly proportional to the concentrations of oxytetracycline employed. The formation of magnesium chelates of oxytetracycline has been reported some time ago (Albert and Rees, Nature 177: 433, 1956) and was readily studied in our laboratory with spectrophotometric methods. Oxytetracycline was actually titrated spectrophotometrically with Mg++ ions with the result that an approximate 50fold molar excess of Mg++ was necessary to saturate the antibiotic. Preincubation of oxytetracycline with Mg++ ions produced maximal inhibitory effects when the oxytetracycline-Mg complex was added to the assay system for code word recognition.

Sedimentation analysis of the oxytetracycline-Mg chelate in the analytical ultracentrifuge with the use of a synthetic boundary cell gave evidence of a molecular weight of the complex of an estimated average of 10,400, indicating a polymer with approximately 20 molecules of oxytetracycline held together by magnesium bridges. A plausible model for the structure of such a polymer would be a stack of twenty oxytetracycline rings with Mg bound either to the carbon 10-11 or the carbon 11-12 enol systems of each adjacent pair of molecules. Such a structure can account for the 1:1 ratio of Mg and oxytetracycline in the complex demonstrated by Albert and Rees (loc. cit.) and for the

nature of the chelating group (Sakaguchi et al., Chem. Pharm. Bull. (Tokyo) 6: 1, 1958).

The hypothesis is being considered and studied that the active form of oxytetracycline is not the metal-free monomeric molecule with a molecular weight of 496 but perhaps a magnesium-chelated polymer of the size and configuration described above. Such a larger complex would offer a multiplicity of hydrogen bonding sites in regular linear array which should facilitate interaction either with messenger-RNA or with transfer-RNA and produce an inhibition of code word recognition such as has been observed.

Summary and Conclusions.

(1) Chloroquine forms a complex with DNA and inhibits biochemical reactions in which DNA must participate as an organizer; these processes are DNA replication and RNA transcription. Exposure to the drug leads secondarily to a massive breakdown of ribosomes in vivo. These biochemical and physiological events explain the killing effects of chloroquine upon microorganisms.

(2) Mepacrine leads to the formation of giant filaments of bacteria owing to an inhibition of DNA synthesis and the ensuing inhibition of cytokinesis; the drug also forms a complex with DNA. Uptake of mepacrine by Plasmodium berghei is readily demonstrable by fluorescence microscopy.

(3) Streptomycin produces a misreading of the genetic code as a corollary phenomenon to the inhibition of protein synthesis.

(4) Chloramphenicol, in contrast, does not influence the reading of the code.

(5) Oxytetracycline inhibits the binding of lysyl transfer-RNA to poly A-complexed ribosomes (an example of the reading of the code); this phenomenon is dependent upon Mg ions. The inhibitory form of oxytetracycline is probably a magnesium-oxytetracycline chelate complex of the molecular weight of the order of 10,000.

Publications.

Hahn, F. E.: Actions of antibiotics on protein synthesis. Proceedings of the IIId International Congress of Chemotherapy 215, 1964.

Wolfe, A. D. and Hahn, F. E.: Mode of action of chloramphenicol IX. Effects of chloramphenicol upon a ribosomal amino acid polymerization system and its binding to bacterial ribosome. Biochim. Biophys. Acta 95: 146, 1965.

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Ciak, J. and Hahn, F. E.: Mode of action of chloroquine. Fed. Proc. 24: 454, 1965.

RESEARCH AND TECHNOLOGY RESUME

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21. TECHNOLOGY UTILIZATION: NA

22. COORDINATION: NA

23. KEYWORDS: Genetics; taxonomy; microorganisms; DNA; antigens; virulence; Salmonella; drug resistance; microbial; Escherichia

24. (U) Tech Objective- The technical objective is to define in genetic and molecular terms in metabolic 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

25. 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 64 - Jun 65) - It has been possible to define the infectious transfer of multiple drug resistance as mediated by a sex factor composed of DNA of variable composition along its length. The results indicate that the drug resistance determin-

26. ants are heterogeneous in origin. Infectious multiple drug resistance was found in a large percentage of drug resistance isolates from the USA, England, Japan and West Germany. It has also been possible to define, as genetic loci, the sites of Vi antigen synthesis in S. typhosa. It is possible to construct Vi containing strains of E. coli and S. typhimurium by recombination. The genetic determinants specifying the virulence of S. typhimurium have been successfully transferred to non-pathogenic mutants of Salmonella. It is hoped that the biochemical basis of virulence may be studied in these strains.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
CFY+1	

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 05, Microbiology

Work Unit 031, Microbial genetics and taxonomy

Investigators.
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Description.

These studies are designed to investigate the genetic characteristics of the metabolic and antigenic changes occurring in enteric bacteria due to genetic recombination, episomic transfer and transduction to clarify the genetics of bacterial virulence and the molecular basis of antibiotic resistance.

Progress.

1. Studies on the Sex Factor of Escherichia coli K-12. The DNA band profile obtained by CsCl density gradient centrifugation of DNA extracted from Serratia marcescens and Proteus mirabilis strains infected with Escherichia coli F-merogenotes shows a native satellite DNA fraction of E. coli density. F-merogenote DNA isolated from these intergeneric hybrids has been examined by the DNA agar technique in an effort to determine its relative size, molecular homogeneity and the relationship between F DNA and that of its host, E. coli K-12.

An F-merogenote (F'-13L) consisting of F and the lac^+ gene cluster was found to be equivalent to 2.5% of the total nucleotide residues of the E. coli K-12 Hfr genome. The size of F alone was estimated to be equivalent to 1.9% of the E. coli genome from the relative degree of homology which exists between F'-13L DNA and the DNA of F-thy^+ and F-gal^+ A substantial portion (almost one half) of the sex factor DNA molecule shows specific duplex formation with the E. coli F^- genome. These homologous regions would presumably provide the necessary pairing to obtain insertion of the autonomous sex factor into the chromosome leading to the Hfr state. Although the formation of specific duplexes between F-DNA and E. coli DNA results from homology in nucleotide sequence, it is not known to what extent these sequences are identical in detail or if they can be considered allelic. It is possible, however, that a large proportion of the homologous region arose from gene pick-up from various hosts during evolution.

The chromosomal material carried by F'-13L was found to be equivalent to 0.4%-0.7% of the E. coli chromosome. This is about three times larger than the size of the only known markers carried by F'-13L which are the lac$^+$ genes. The chromosomal segment carried by F'-13L, therefore, is not restricted only to the loci which play a part in lactose utilization, but certainly the number of functional markers is small.

The DNA of F'-13L is separable into two general components upon temperature fractionation. The majority of the F-merogenote specific DNA duplexes exhibit a mean composition of 50% G+C but about 10% of the duplexes show a mean composition of 44% G+C.

Our concept of the sex factor DNA is a continuous structure of variable composition along its length. We picture the sex factor genome as being divided into three regions; (a) A region comprising about 40% of the sex factor genome which is homologous with a limited fraction of the E. coli genome and probably concerned with episome-host interactions. (b) A region comprising about 50% of the sex factor genome which is not homologous with the E. coli genome and which is probably concerned with purely episomal functions. (c) A region of about 10% of the sex factor genome, presumably also non-homologous, with an overall base composition of 44% G+C, possibly representing a specific functional region of F.

2. Studies on Infectious Multiple Drug Resistance in Enteric Bacteria. We have previously reported (Annual Report, 1963-1964) that R factors consisting of an episome, RTF, and carrying drug resistance markers may be transmitted among enteric bacteria by conjugation. DNA extracted from Proteus cells infected with R factors from E. coli has been examined in CsCl density gradients. During the past year we have extended our preliminary impression that a correlation exists between the genetic constitution of R factors and the molecular banding pattern of their DNA in CsCl density gradients. We have found that the chloramphenicol CM resistance marker of R factors is composed of high G+C base pairs (56% G+C average) while the tetracycline (TC) marker is composed of high AT base pairs (48% G+C average). The sulfonamide SU and streptomycin SM drug markers carried by R factors are very closely linked genetically and on the molecular level exhibit essentially equimolar base pairs. The kanamycin-neomycin marker of R factors is associated with a DNA molecule having an average G+C composition of 54%.

Naturally occurring R factors differ not only in regard to their drug resistance markers, but also with respect to their supression of the sex factor, F, of E. coli K-12. R factors which suppress the function of F are called fi$^+$; those that do not are called fi$^-$. The fi$^-$ R factors, when present in E. coli strains, reduce the efficiency of plating of phages λ and T1 whereas fi$^+$ R factors in the same strains do not exhibit this inhibiting action. Superinfection immunity and mutual exclusion were found between fi$^+$ R factors but not between fi$^+$ and fi$^-$ R factors. Moreover, fi$^+$ and fi$^-$ R factors do not genetically recombine, but

different fi$^+$ R factors do so easily. Finally, all naturally occurring R factors so far studied have lacked the CM determinant.

It has been concluded on the above genetic grounds that fi$^+$ and fi$^-$ R factors are considerably different elements. It seemed advisable, therefore, to examine a series of fi$^+$ and fi$^-$ R factor DNA preparations to determine if these genetic differences were, in any way, reflected in molecular terms. We have now examined a total of 14 independent R factor isolates recovered from a variety of sources and geographical locations. Despite the wide differences in geographical isolation (USA, Japan, W. Germany and England) and strain source (E. coli, Shigella sp., S. typhimurium and S. newport), those with identical resistance determinants and fi characteristics appear quite similar to their banding patterns in CsCl. Such results suggest a common ancestry for these R factors.

We have not been able to determine any meaningful gross molecular difference between fi$^+$ and fi$^-$ R factors. Since all naturally occurring fi$^-$ R factors were found to lack the CM drug marker, we suspected that CM and fi$^+$ might be closely linked. Our results clearly show that the CM resistance determinant is associated with 56% G+C DNA molecules. It is possible to isolate fi$^+$ R factors which do not carry CM resistance and in these cases there is no detectable 56% G+C component. Moreover, we have isolated segregants from an fi$^+$ R-TC, SU, SM, CM R factor which has retained only R, TC and fi$^+$ and this segregant has no trace of a 56% G+C component. It seems probable, therefore, that the fi determinant is carried on the RTF component and very likely has a different overall base composition from that of the CM marker. Yet, the general association of the CM resistance determinant only with the fi$^+$ state suggests that the fi$^+$ determinant may be a necessary concommitant for the acquision of the CM marker by the episome.

Our results indicate that the RTF component of R factors is composed of DNA of an average composition of 50% G+C -- essentially identical to that of the E. coli and Shigella genomes in which the R factors were first demonstrated. This composition is also essentially that of the E. coli sex factor which determines maleness in E. coli K-12. The genetic functional and physical similarities between the sex factor and RTF suggests that these two conjugation factors may be related. We are presently examining this matter and genetic studies indicate that F and R undergo recombination. If, as it seems, RTF was ancestrally like F, we are faced with the problem of explaining the origin of the multiple drug resistance determinants subsequently acquired by RTF. By analogy with F, the most likely hypothesis is that RTF acquired the resistance determinants by gene pick-up from the chromosomes of resistant bacteria. Yet, the type of drug resistance associated with R factors is apparently unrelated to that previously described for naturally occurring drug resistant enteric organisms; nor have allelic counterparts for the drug resistant determinants of R factors been thus far detected in any bacterial species. In addition, it seems unlikely that the loci determining re-

sistance to four or five different drugs would be so fortuitously linked together on the bacterial chromosome to be acquired in a single step by RTF. The two likely possibilities which fit all of the observed data are: (a) that gene pick-up by RTF occurred in a series of independent recombination events and (b) that the drug determinants were acquired by different RTF factors and multiple factors were formed by recombination in cells simultaneously carrying different R factors.

3. R Factors From Army Medical Installations. Multiply drug resistant enteric species have been submitted from several military installations to this laboratory to determine if R factors were present in their areas. Sixteen cultures of E. coli were submitted by WRGH and one strain was found to harbor an R-SU, SM, TC, CM factor. The Sixth Army Medical Laboratory submitted four strains of S. newport and one of S. flexneri which were multiply drug resistant. The S. newport strains all harbored an R-SU, SM, TC factor while the S. flexneri strain harbored an R-SU, SM, TC, CM factor. Finally, 44 cultures of multiply resistant enteric species isolated from diarrhea cases in Viet Nam were examined for the presence of R factors. Of the 44 cultures, 21 were found to harbor R factors. Five E. coli strains and one strain of S. flexneri 1b were found to harbor an R-SU, SM, TC, CM R factor, while seventeen S. flexneri 1b cultures were found to harbor an R-SU, SM, TC factor.

4. Episomic Elements in Proteus. All wild type Proteus strains are lactose negative, however, a lactose fermenting strain has been isolated from hospital patients. The ability of this strain to ferment lactose has been shown to be associated with the presence of an episome, which is called P-lac. Ultracentrifugal analysis has shown that the DNA extracted from this Proteus strain contains an unusual minor component, amounting to 10% of the total. This minor component has a 50% G+C content and differs markedly from the 39% G+C content of normal Proteus DNA.

The P-lac episome is quite stable in Proteus and the DNA must be continually replicated to be maintained in the cell. This DNA of a different base composition can be maintained with no apparent harm to the cell and, in addition, the cell can use this DNA to make the enzymes necessary for the fermentation of lactose. The inducible enzymes associated with lactose fermentation in E. coli are also present in Proteus. The three enzymes studied were β-galactosidase, a lactose splitting enzyme; galactoside permease, an inducible enzyme which concentrates substrate in the cell; and thio galactoside transacetylase, an enzyme of unknown function which is genetically linked to the permease. The following table shows the levels of enzyme production by the Proteus strain with the P-lac episome before and after induction compared to the enzyme levels in a wild type E. coli K-12 strain shown in parenthesis.

TABLE 1

ENZYME PRODUCTION BY PMI P-lac

Inducer	β-galactosidase (EU/mg protein)		Permease (% accumulated)		Transacetylase (EU/mg protein)	
None	6	(0.2)	0.3	(0)	1.9	(0)
10^{-3}M TMG	15	(85)	1.1	(1.5)	3.2	(8.3)

The control mechanism which regulates enzyme synthesis in the uninduced E. coli does not function properly in Proteus because there is a definite basal level of enzyme produced even before induction, also the induced levels of these three enzymes are less in Proteus than in E. coli.

Similar results are also obtained when the F-lac episome is transferred to Proteus. This implies that genetically the lactose operon of the P-lac episome is also complete and whatever defect that exists in the regulation of enzyme production must be a defect in the host Proteus cell.

The next table shows the level of β-galactosidase production when the P-lac and F-lac episomes are transferred to other bacterial species which are typically lactose negative like Proteus.

TABLE 2

β-GALACTOSIDASE SYNTHESIS
(EU/mg protein)

	Strain	No inducer	10^{-3}M TMG
Proteus mirabilis	PM1	0	0
" "	PM1 F-lac	5	11
" "	PM1 P-lac	6	15
Serratia marcescens	SMS^r11	0.5	0.5
" "	SMS^r11 F-lac	0.5	36
" "	SMS^r11 P-lac	0.5	29
Salmonella typhosa	643	0.2	0
" "	643 lac^+	0.2	55
" "	643 F-lac	0.2	105
" "	643 P-lac	0.3	98
E. coli K-12	W3876 F-lac	0.4	194
" " "	W3876 P-lac	0.3	172

All these strains show a lower level of synthesis than E. coli with the episome, but only Proteus has the defect in regulation.

Characterization of the β-galactosidase from Proteus indicated that the enzyme produced by cells with the P-lac episome was different from the normal coli β-galactosidase. These differences are reflected in the immunological behavior of the enzymes and in the greater heat sensitivity of the enzyme produced by strains with the P-lac episome. Purification procedures using columns of Sephedex G-200 and DEAE-Sephedex have been used to obtain the β-galactosidase in pure form from this Proteus strain and this enzyme is now being analyzed for differences in amino acid content.

We have previously shown the episomic element F-lac to be transferrable from Escherichia coli (50% G+C) to Proteus mirabilis (39% G+C) despite their divergent DNA base compositions. When this episome is present in Proteus, reduced levels of enzymes of the lactose operon result compared to those attained by E. coli. We have now been able to demonstrate transfer of the episome F-gal from E. coli to a galactose negative (gal$^-$) mutant of P. mirabilis at a frequency of 10^{-6} per donor cell. Although considerably more unstable than in E. coli, F-gal in Proteus can be easily retained by selective culture using galactose as sole carbon source. Transfer of F-gal from P. mirabilis F-gal donors back to E. coli gal$^-$ strains occurs at frequencies of from 1^{-5} to 10^{-6} per donor cell. DNA preparations from the parent Proteus strain prior to infection with F-gal and from recombinants of Proteus receiving F-gal were examined by CsCl density gradient ultracentrifugation. Appearance of a satellite DNA band with a density equivalent to 50% G+C was observed only in the P. mirabilis strains containing F-gal. The satellite band associated with the presence of F-gal is estimated to be about 2% of the total DNA. F-gal appears to function unimpaired in Proteus insofar as galactokinase production is concerned.

5. Genetic Mapping of Vi and Somatic Antigenic Determinants in Salmonella. Previous genetic studies of Salmonella antigens have centered principally around the flagellar or H-antigens of these species, and such studies have employed only transductional techniques. With the isolation in this laboratory of a Salmonella typhosa Hfr strain capable of transferring chromosomal material to Salmonella typhimurium recipients (Annual Report, 1963-1964), it has been possible to study these two organisms with regard to the genetic relationship of their somatic as well as their flagellar antigens. In addition, an opportunity was afforded to examine the genetic basis of the Vi antigen of S. typhosa.

The Vi antigen was transferred to a number of S. typhimurium recipients by mating with the S. typhosa Hfr strain TD-7. In these crosses, unselected genetic markers were inherited in significant numbers with the selected gene only when the determinants in question were closely linked. It was possible, therefore, to exploit this situation for the purpose of mapping both the Vi and the somatic 9 antigenic determinants of S. typhosa. Some of the principal crosses performed are shown in Table 3. It will be noted that in the selection for the distal donor

marker, his$^+$ (cross 1), most proximal unselected markers are inherited at only 1 to 10%. Thus, the fact that 80% of this hybrid class received the somatic antigen 9 of TD-7 immediately established the close linkage of the somatic antigen 9 gene (0-9) with his. Further, all hybrids receiving 0-9 concurrently lost their native somatic antigen 4, suggesting the possible allelism of the 0-9 and 0-4 genes. Inheritance of the Vi antigen exceeded 6% in only two instances: 33% when the selected marker was pur-A$^+$, and 18% when the closely linked pyr-B$^+$ marker was selected. These percentages, plus those for inheritance of the closely linked inos$^-$ gene (crosses 3 and 4) make it possible to map the Vi determinant in relation to the known inos-pur-A-pyr-B linkage group. The order is Vi - inos - pur-A - pyr-B.

The probable allelism of the 0-4 and 0-9 genes was confirmed by mating a Vi negative histidine requiring (his$^-$) S. typhosa recipient with the S. typhimurium Hfr strain, B-2. In this cross, where unselected marker inheritance ranged from 2 to 10%, 73% of the his$^+$ S. typhosa hybrids received the 0-4 antigen of the donor, and concurrently lost 0-9. Although neither parent strain, in this instance, possessed the Vi antigen, the Vi positive character was found to segregate among the hybrids, with a significant 25% of the his$^+$ hybrid class acquiring the Vi antigen. This indicated the presence of a second Vi antigen determinant, located near his, which is common to both S. typhosa and S. typhimurium.

The high percentages of 0-9 and 0-4 gene inheritance with the his gene strongly suggested the feasibility of their cotransduction with this marker. Therefore, phage PLT-22 grown on a S. typhimurium hybrid which had received his$^+$ and 0-9 from TD-7 was used to transduce the his$^+$ gene to a his$^-$ S. typhimurium recipient. Transduction of his$^+$ occurred at a frequency of 10^{-6} with 24% of the transductants also receiving 0-9. All transductants receiving 0-9 concurrently lost 0-4. Cotransduction of his$^+$ and 0-4 was accomplished with a PLT-22 lysate of S. typhimurium using a his$^-$ strain of S. typhosa as recipient. Again, 21% of the his$^+$ transductants also inherited 0-4 and concurrently lost 0-9.

TABLE 3

UNSELECTED MARKER INHERITANCE BY THE PROGENY OF CROSSES BETWEEN *S. TYPHOSA* Hfr TD-7 AND *S. TYPHIMURIUM* LT-2 RECIPIENT STRAINS

Cross	Selection	Per cent unselected markers*							
		ara$^-$	*inos*$^-$	Vi$^+$	*met*-A$^+$	*rha*$^-$	*fuc*$^-$	*som* 9	*his*$^+$
(1) TD-7 X HMXS^r	*his*$^+$	10	5	3	1	3	6	80	ALL
(2) TD-7 X HMXS^r	*met*-A$^+$	1	2	2	ALL	4	<1	<1	<1
(3) TD-7 X Pur-AS^r	*pur*-A$^+$	-	42	33	-	N.T.	N.T.	N.T.	-
(4) TD-7 X Pyr-BS^r	*pyr*-B$^+$	6	30	18	-	N.T.	N.T.	N.T.	-
(5) TD-7 X PanS^r	*pan*$^+$	31	5	6	-	N.T.	N.T.	N.T.	-

* Percentages are based on a minimum of 105 tested hybrids of each selection class.

N.T. = Not Tested.

6. Genetics of Virulence in Salmonella. We have continued to investigate genetic mating systems in Salmonella typhimurium which make possible the study of extensive regions of the chromosome. Previously, virulent strains of S. typhimurium were rendered avirulent by recombination with avirulent S. abony donors and two additive genetic loci were associated with loss of virulence.

Further matings have been undertaken to determine whether virulence characteristics can be transferred from virulent S. typhimurium donors to avirulent S. typhimurium recipients. A well-marked recipient strain of S. typhimurium, strain SW 1292B which is negative for the markers pro, leu, ara, mal, rha, xyl, fuc, and gal was crossed with the highly virulent (LD_{50}=10 organisms in saline) Hfr A strain of S. typhimurium. Hybrids were selected for pro^+ leu^+, and the other selective markers ara^+, mal^+, rha^+, xyl^+, fuc^+ and gal^+. The hybrids were purified and their unselected markers were determined in order to establish the extent of the chromosomal segment transferred from the virulent donor to the avirulent recipient. The results obtained strongly indicated that only those hybrids which received the gal^+ region of the chromosome showed increased degrees of virulence from the parental virulence level of SW 1292B of $LD_{50}=5x10^7$ ($LD_{100}=1x10^8$; $LD_0=5x10^6$). Increased virulence levels in the hybrids ranged from LD_{50}'s of $1x10^5$ to instances of complete acquisition of the virulence of the donor parent (LD_{50}=10-100 organisms).

A number of hybrids were isolated in further crosses which were characterized as having received only the gal^+ region from the virulent donor. Among these hybrids, increases in virulence occurred including some with the complete virulence level of the donor. These results would indicate that the avirulence of the S. typhimurium strain SW 1292B is probably due to a single mutation in a gene linked to the gene for galactose utilization.

Summary and Conclusions.

1. An F-merogenote consisting of F and the lac^+ gene cluster was found to be equivalent to 2.5% of the E. coli K-12 Hfr genome. The size of F alone was estimated to be equivalent to 1.9% of the E. coli genome. Almost one-half of the sex factor molecule shows a relationship to the E. coli F^- genome. The majority of F specific duplexes exhibit a mean composition of 50% G+C but almost 10% of the duplexes show a mean composition of 44% G+C.

2. The satellite DNA associated with a number of R factors and R factor variants following this transfer to P. mirabilis has been examined. The spontaneous segregation of single or multiple drug resistance was associated with a reduction in the size of the satellite DNA. It is concluded that the loss of drug determinants by R factors most probably is a physical deletion of genetic material due to incomplete replication.

The DNA loss associated with the segregation of drug determinants was not random, but rather, specific density regions of the R factor DNA were found to be reduced or lost. Naturally occurring R factors may be differentiated genetically as *fi*$^+$ or *fi*$^-$ on the basis of their restriction of the *E. coli* sex factor, modification of phages and superinfection immunity. There is no definite correlation between the banding of an R factor in CsCl and the *fi* state. However, the general association of the chloramphenicol resistance determinant and the *fi*$^+$ state suggests that *fi*$^+$ may be a necessary concommitant for the acquisition of the chloramphenicol marker by the episome. R factors have been isolated from throughout the world and in a variety of bacterial species; those with identical resistance determinants and *fi* characteristics appear quite similar both genetically and in their banding pattern in CsCl. Such results suggest a common ancestry for these R factors.

3. R factors have been detected in cultures submitted from military installations in the USA and from Viet Nam.

4. The presence of a lactose episome in *Proteus* results in the synthesis of a β-galactosidase, a permease and transacetylase which the cell did not produce before. However, the regulatory mechanism of the lactose operon which operates in other lactose positive strains does not operate properly in *Proteus*. The level of production of these enzymes in a fully induced culture of *Proteus* are much less than in *E. coli* and reductions are also seen when the episomes are transferred to *Salmonella* and *Serratia* strains. F-*gal* appears to function as well in *Proteus* as does the wild-type *gal*$^+$ gene of *Proteus*.

5. *Salmonella typhosa* Vi and somatic 9 antigens were transferred to *S. typhimurium* recipient strains by mating, and the genes responsible for these antigens were mapped. Two genes were found to determine Vi antigen expression. One of these was mapped near the *inl-pur-A-pyr-B* linkage group and the other was found to be situated near *his*. The gene determining somatic antigen 9 specificity was found to be closely linked to, and cotransducible with, the *his* gene. The somatic antigen 9 determinant was shown, by conjugation and transduction experiments, to be allelic with the somatic antigen 4 gene of *S. typhimurium*.

6. Restoration of virulence to an avirulent strain of *S. typhimurium* has been accomplished by mating with a virulent donor *S. typhimurium*. The restored virulence gene is linked to the *gal*$^+$ gene.

Publications.

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Bacteriol. Proc. (Am. Soc. Microbiol.) p 14 (1965).

PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08
Physiology

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6437	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 65	A. NEW	U / U	NA	QR	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71P 08 075

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11. TITLE:

(U) Cell growth and regeneration (21:09)

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
016200 Stress physiology; 012900 Physiology; 002300 Biochemistry	09 58	NA	OTHER | DA

16. PROCURE. METHOD	17. CONTRACT/GRANT a. DATE / b. NUMBER / c. TYPE / d. AMOUNT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	NA	PRIOR FY 65	4	37
		CURRENT FY 66	4	40

19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL: 202-576-5284 TYPE: DA

21. TECHNOLOGY UTILIZATION: Clinical medicine

22. COORDINATION: NA

23. KEYWORDS: Cell physiology; physiology; cell division; tissue culture; growth; regeneration; adenosinetriphosphate; nucleic acids; protein

24. (U) Tech Objective - The definition of the molecular systems regulating cellular growth and regeneration following injury such as mechanical, thermal or chemical trauma, infection, ionizing radiation and shock. It is anticipated that eventual clinical application of the information obtained will greatly enhance the means of protection and therapy in the types of injury mentioned.

25. (U) Approach - To maximize experimental control, accuracy of quantitation, and analytical resolution, a biphasic logarithmic-stationary mammalian cell culture system was developed. This system was shown to respond to injury in a manner closely approximating the response of body tissues. Thus, cell loss is followed by compensatory cell growth and regeneration and gamma-irradiation by a limited number of cell divisions with subsequent loss of the reproductive ability of the cells. To uncover the molecular interactions underlying these responses a detailed analysis of cellular metabolism correlated with cell population kinetics was planned.

26. (U) Progress (Jul 64 - Jun 65) - Normally fed and starved logarithmic cultures were compared with stationary cultures. While both log starved and stationary populations showed inhibition of cell division and low ATP levels, the former were characterized by low levels of DNA, RNA, and protein while in the latter these constituents were normal. Thus, ATP appears to be limiting for cell division in both instances, but in contrast to the simple nutritional deficiency of the log phase, the operation of a regulatory mechanism in the stationary phase is apparent. This mechanism is currently under investigation.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands) CFY+1	36.

DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

PROJECT 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08 Physiology

Work Unit 075, Cell growth and regeneration

Investigators.
Principal: Andre D. Glinos, M.D.
Associate: Capt Robert E. Maxwell, MC; Edwin M. Bartos, Ph.D.; Hanson H. North, B.S.; Robert J. Werrlein, B.S.

Description.

The available evidence indicates that cell division and proliferation is the determining factor in the following situations of Military Medical interest: (a) wound healing, (b) radiation injury, (c) infection, and (d) transplantation.

(a) In Wound Healing following mechanical, thermal or chemical trauma, regeneration and repair of lost tissue depends on the controlled cellular division and multiplication of epithelial and mesenchymal elements, the latter being responsible for the elaboration of the intercellular matrix substances such as collagen.

(b) In Radiation Injury it has been well known for years that the lesion is mainly located in the rapidly dividing and proliferating cells. Recent work has shown that interactions between changes induced at the time of irradiation and the processes of post-irradiation cellular growth and division account for the extensive injury of proliferating cells. Such injury when located in the cells discussed in the next paragraph is in turn responsible for the high susceptibility to infection of the irradiated host.

(c) In Infection it has been well known for years that the first defense response of the host is the division and proliferation of the stem cells of polymorphonuclear leukocytes capable of phagocytosis. The recent revolution in thinking regarding infection consists of the demonstration that the immune process is also one of cellular division and proliferation, involving a complex of cells capable of producing antibody, in response to antigenic stimulation.

(d) In Tissue Transplantation the response of the host and the fate of the graft is determined by the extent of division and proliferation of cells capable of responding to tissue antigens in a manner similar to the one described in para (c) for infection.

Knowledge of the mechanisms controlling cell growth would seem therefore to be the most essential prerequisite for the eventual mastery and control of all clinical situations related to mechanical, thermal or chemical trauma, radiation injury, infection and transplantation.

Progress.

Analysis of the mechanisms controlling the growth of tissue cells in situ is greatly handicapped by inadequate control of the cellular environment and limited accessibility of the cellular site resulting in inadequate sampling, quantitation and replication. Earlier work on liver regeneration in situ although it led to the formulation of concepts related to cell growth control and, as a by-product, even carcinogenesis, has clearly delineated these limitations. To overcome these difficulties and maximize experimental control, accuracy of quantitation and analytical resolution, mammalian cell populations growing in vitro are being used. Previous work (see 1964 Progress Report of this work unit) has shown that under the proper conditions these populations respond to injury in a manner closely approximating the response of body tissues. Thus, populations in the stationary phase respond to cell loss by cell division and proliferation. When the original population is restored cell proliferation stops, exactly as in the regeneration of body tissues following injury. The response of this cell culture system to radiation injury was also shown to approximate the response of body tissues and in addition provided some clues regarding the molecular systems controlling cellular growth and proliferation.

During the current period this work was continued with the emphasis placed on the molecular interactions involved in the transition of a cell population from the active log growth phase to the stationary phase. The term stationary phase has been used to describe generally the terminal plateau exhibited by the growth curve of a bacterial or a mammalian cell culture if no dilution and subculture takes place. While the secretion into the medium of growth inhibitory substances by the cells has been suspected in many instances, no such substances have ever been isolated. On the other hand, it is known that in a suspension culture with periodic renewal of the medium by centrifugation and resuspension of the cells in new medium the cell density where the plateau occurs depends on the frequency of medium renewal. Based on this fact it has been hypothesized that the occurrence of the plateau is essentially a manifestation of a nutritional deficiency, the cells being in a state of starvation or semi-starvation. Using this proposition as a springboard for the investigation of the molecular interactions involved in the induction of the stationary phase, suspension cultures of the L strain mouse fibroblasts were grown with and without renewal of the medium and the resulting growth curves are shown in Fig. 1. The medium used in both types of culture was Eagle's Minimum Essential Medium supplemented with 10% horse serum and by

growing the cultures in a CO_2 incubator the pH was kept within 7.10 and 7.30 throughout the duration of these experiments. It can be seen that following an initial log growth period both cultures reached a plateau phase. In the case of the culture without medium renewal the plateau occurred when cell density reached 15 x 10^5 cells/ml after 48 hours of incubation and lasted until 84 hours at which time the population started declining. In the case of the culture with daily medium renewal the plateau occurred when cell density reached 70 x 10^5 cells/ml after 15 days of incubation and the cell population showed no signs of decline until the 22nd day when the culture was used for a number of determinations. In a great number of replicate experiments no evidence has been obtained that the plateau phase of a culture with daily medium renewal is limited in time while without medium renewal the plateau phase invariably came to an end between 84 and 96 hours. As this latter event was obviously due to nutritional deficiency the plateau phase of cultures without medium renewal will henceforth be referred to as starvation plateau, the term stationary phase being reserved for cultures with daily medium renewal. Table I shows that this distinction between the two types of plateau is also reflected in the constitution of the cells in terms of their volume, measured electronically with the Coulter counter, their DNA and RNA content, measured by the method of Scott, Fraccastoro and Taft, their protein content measured by the method of Oyama and Eagle, and in their viability in terms of their plating efficiency, estimated from the per cent of cells capable of forming colonies upon subculture in fresh media. In the case of the culture without medium renewal these measurements were carried out at the beginning of the log phase at 0 time and at the beginning and the end of the plateau phase, at 48 and 84 hours respectively (Fig. 1, lower curve). In the case of the culture with daily medium renewal, these measurements were carried out at 0, 12 and 24 hours during the last twenty four hour interval of the stationary phase between day 23 and 24 (Fig. 1, upper curve). It can be seen that during the starvation plateau, cell volume and DNA, RNA, and protein levels declined sharply. The loss of such key cellular components led in turn to a decline of cell viability as indicated by the decrease of the plating efficiency of the cells from 50 to 28 per cent. There is a sharp contrast between these findings and the findings in the stationary phase where measurements over a 24-hour period showed a remarkable constancy of the constitution and viability of these cells, at levels identical with the levels characteristic of the beginning (0 time) of log growth. In order to obtain some clues as to the mechanisms involved in the transition of a cell population from the logarithmic phase of growth to the starvation plateau on one hand and to the stationary phase on the other, cell division together with four parameters related to cellular energy production were investigated next. The results obtained are shown in Table II. Cell division was estimated by counting the per cent of cells in mitosis after appropriate [illegible]ion and staining of the cells, ATP levels representing the intracellular stores of energy immediately available for

synthesis were determined by the luciferin-luciferase reaction of Strehler and Totter, medium glucose levels representing the main exogenous energy supply were determined by the glucose oxidase method, medium lactate representing the end product of glycolysis and a potential source of energy by the chemical method of Barker and lactic dehydrogenase (LDH); a key enzyme for lactate production and utilization was determined by the enzymatic method described by Bergmeyer. Data obtained at 12 and 84 hours for the log and starvation plateau phases, respectively, and data obtained at 12 and 24 hours during the last twenty-four hour interval of the stationary phase cultures (c.f. Fig. 1) are presented, because serial determinations as a function of time showed these specific time intervals to be the most representative of the phases under study. The data of Table II reveal one striking similarity and one equally striking dissimilarity between the starvation plateau and stationary phases. The similarity is that, in contrast to the high mitotic activity and ATP level of the logarithmic phase at 12 hours, both starvation plateau and stationary phase cultures show almost identical low levels of mitotic activity and ATP. The dissimilarity is that lactate continues to accumulate during the starvation plateau following log growth and LDH activity is low while in the stationary phase the lactate accumulated during the first 12 hours is utilized extensively during the following 12 hours and LDH activity is ten times higher than in the starvation plateau.

These findings together with the data shown in Table I suggest that the intracellular supply of energy in the form of ATP is an important factor in regulating cell division in both starvation plateau and stationary phase cultures. However, while in the case of the starvation plateau, this low ATP level is obviously the result of simple nutritional deficiency this is not the case in the stationary phase (c.f. Table I) where the high LDH activity level and the extensive utilization of lactate (c.f. Table II) indicate a markedly different mechanism for ATP production. The molecular interrelations suggested by these findings are currently under investigation and constitute the basis for further work on the regulation of cellular growth and regeneration following injury.

Summary and Conclusions.

The objective of this work unit is the definition of the molecular systems regulating cellular growth and proliferation following injury such as mechanical, thermal, or chemical trauma, irradiation, infection; and in tissue transplantation. It is anticipated that eventual clinical application of the information obtained will greatly enhance the means of protection, therapy and control in the conditions mentioned.

To maximize experimental control, accuracy of quantitation, and analytical resolution, a biphasic logarithmic-stationary mammalian cell culture system was developed. This system was shown to respond to

injury in a manner closely approximating the response of body tissues. Thus, cell loss is followed by compensatory cell growth and regeneration and gamma-irradiation by a limited number of cell divisions with subsequent loss of the reproductive ability of the cells. To uncover the molecular interactions underlying these responses a detailed analysis of cellular metabolism correlated with cell population kinetics was planned.

Normally fed and starved logarithmic cultures were compared with stationary cultures. While both starved and stationary populations showed inhibition of cell division and low ATP levels, the former were characterized by low levels of DNA, RNA, and protein while in the latter these constituents were normal. Thus, ATP appears to be limiting for cell division in both instances but in contrast to the simple nutritional deficiency of the log phase the operation of a regulatory mechanism in the stationary phase is apparent. This mechanism is currently under investigation.

Publication

Glinos, A. D.: On the applicability of the two-stage concept of initiation and promotion to chemical carcinogenesis in the liver. Acta, Un. int. Cancr., 20: 571-572, 1964.

F I G U R E 1

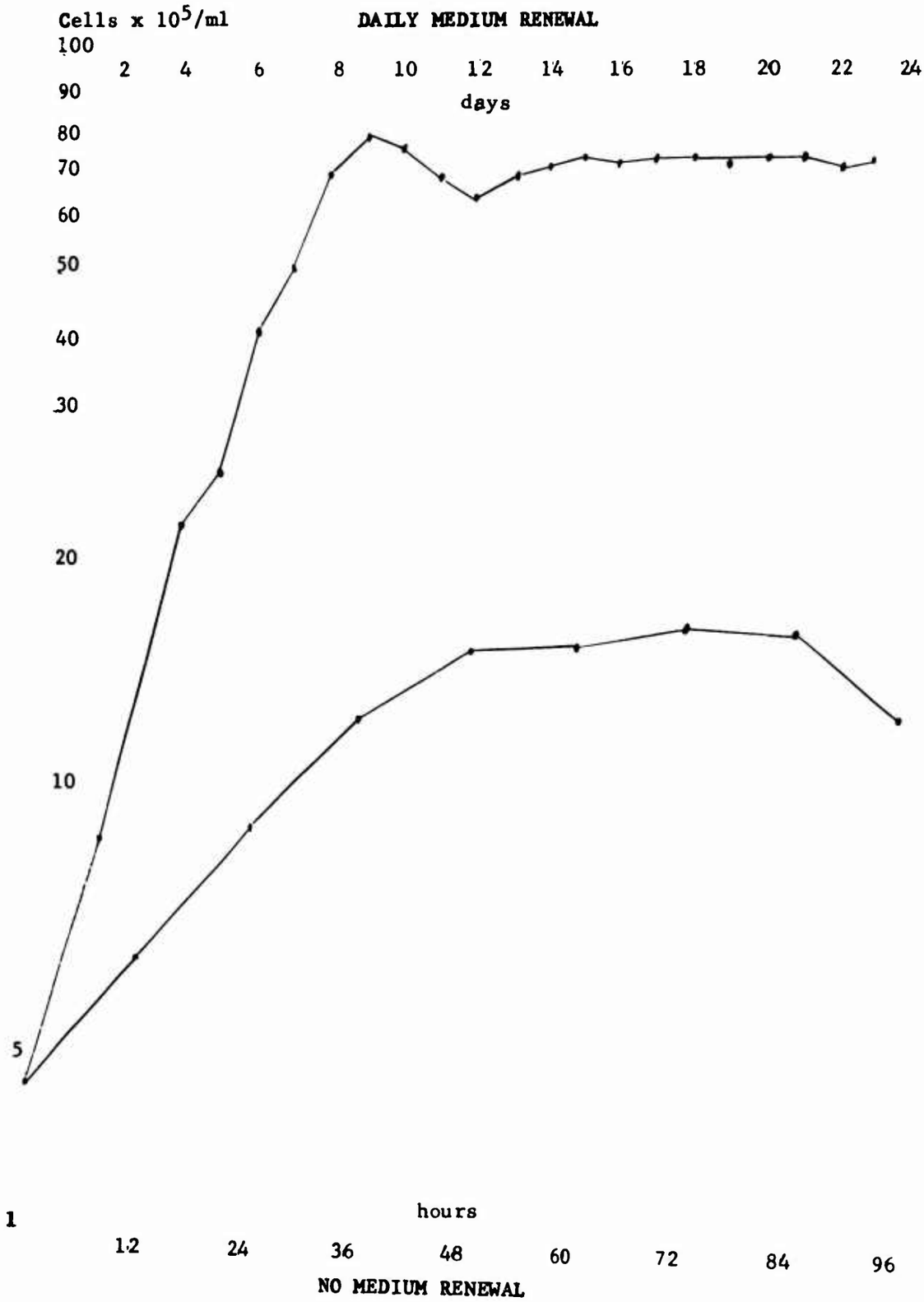
Cells x 10^5/ml
DAILY MEDIUM RENEWAL
100
90
80
70
60
50
40
30
20
10
5
2
4
6
8
10
12
14
16
18
20
22
24
days
1
hours
12
24
36
48
60
72
84
96
NO MEDIUM RENEWAL

TABLE I

	Log growth and Starvation plateau			Stationary phase		
Hours	0	48	84	0	12	24
Mean cellular volume μ^3/cell	1414	1228	796	1367	1496	1459
Mean DNA content $\mu g \times 10^{-6}$/cell	24	23	14	23	22	28
Mean RNA content $\mu g \times 10^{-6}$/cell	41	38	18	34	36	33
Mean protein content $\mu g \times 10^{-6}$/cell	336	324	237	301	365	363
Plating efficiency % of cells forming colonies	53	50	28	--	--	51

TABLE II

	Log growth and Starvation plateau		Stationary phase	
Hours	12	84	12	24
Per cent of cells in mitosis	1.20	0.05	0.20	0.05
ATP $\mu M \times 10^{-9}$/cell	6.88	2.28	2.34	2.40
Glucose μg/ml of medium	808	100	126	0.00
Lactate μg/ml of medium	168	419	340	143
Lactic dehydrogenase units $\times 10^{-6}$/cell	165	170	1829	1855

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24. (U) Tech Objective - Morphological analysis of the primate and subprimate CNS directs mainly toward delineation of longitudinal systems. An analysis of structures supplied by the autonomic nervous system in regards to method of CNS control.

25. (U) Approach - Determination of CNS pathways and terminations in various primates, birds and reptiles by the Nauta technique following appropriate surgical lesions. Recording and analysis, utilizing computer techniques, of the cardiovascular and electrocortical response to controlled hemorrhagic shock in awake chronic monkeys. Major problems include instrumentation of the monkeys and computer programming for the massive data being collected.

26. (U) Progress (Jul 64 - Jun 65) - Determination of CNS pathways has been expanded to the chimpanzee. In the other animals additional fiber tracts have been plotted. The technical problems encountered in instrumenting the monkeys for the hemorrhagic shock experiences have been mostly overcome. Progress has been made in the satisfactory recording of physiological data in digital form for best utilization of existing computers.

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 076, Analysis of behavior and of mediating mechanisms: Anatomic and electrophysiological factors

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Description: The general object of this subtask is the analysis of neural mechanisms mediating behavior in the widest sense. Within the frame of this purpose the specialized techniques of anatomy, physiology, experimental psychology and endocrinology are (by necessity) all used. Since some aspects of behavior can be studied in greatest detail within a specific discipline much of the work carried out has been done using the classical methods of these disciplines. However, many aspects of behavior can be studied only when the classical approaches are extended and combined with newer techniques from other disciplines. It will be evident from this report that considerable effort is presently being put into interdisciplinary approaches to specific behavioral problems. These pioneer projects, besides offering new methods of attack on different subjects, also serve to strengthen the background of the investigators involved and aid in better conceptualization of the basic problems to be studied in the analysis of behavior.

Efferent connections of the neocortex. During the past year the main emphasis of this experimental-anatomical study has been on the subcortical projections arising from the precentral and postcentral gyri in the rhesus monkey and chimpanzee, and of the superior and inferior parietal lobules (areas 5 and 7) in the rhesus monkey. Particular attention was paid to the cortical connections with the basal ganglia, diencephalon and mesencephalon. The precentral gyrus was found to project to the caudate nucleus, putamen and claustrum and the thalamic nuclei ventralis lateralis, reticularis and centralis lateralis. The rostral subdivision of the gyrus (area 6 of Brodmann) has additional projections to the nuclei paracentralis, medialis dorsalis and parafascicularis; the caudal subdivision, Brodmann's area 4, projects to

centrum medianum. The gyrus also has additional connections with the subthalamic region (fields H_1 and H_2 of Forel and the zona incerta) and the pontine nuclei (pons Varolii), prerubral field and red nucleus of the mesencephalon. Postcentral cortical fiber projections (areas 3-1-2 of Brodmann) were found in the caudate nucleus, putamen and claustrum and the thalamic nuclei ventralis posterior and reticularis. In the subthalamic region only a few fibers were found in the zona incerta and fields of Forel. In the mesencephalon fibers were traced to the pontine nuclei (pons Varolii), prerubral field and red nucleus. The subcortical connections arising from the precentral and postcentral gyri have also been traced to the hindbrain and spinal cord. Analyses of the subcortical projections from areas 5 and 7 of the parietal lobe are now in progress. This study has been expanded to include an anatomical analysis of the cortical projections in the marsupial (Virginia opossum) and the corticospinal projection in two prosimian primates, a lorisiform lemur (Nycticebus) and the tree shrew (Tupia). It was found that the corticospinal fibers in the lemur enter the contralateral lateral funiculus and descend the length of the cord. These fibers were distributed in abundance to the dorsal horn and were found also in the zona intermedia and ventral horn. In the tree shrew, corticospinal fibers descend bilaterally in the dorsal funiculi, the major part of this projection distributing to the contralateral dorsal horn. The tract could be followed into thoracic segments. Corticospinal fibers in the opossum descend in the dorsal funiculus and reach thoracic segments, and were traced to a termination in the dorsal horn. Direct corticospinal connections with motor ventral horn cells were seen only in the slow loris Nycticebus.

Efferent connections of the cerebellum. Experimental anatomical studies were begun in the rhesus monkey in order to compare the efferent fiber connections of the cerebral cortex and cerebellum to diencephalic, midbrain and hindbrain nuclei. Major emphasis will be paid to cerebello-diencephalic connections. Stereotaxic coordinates were established and lesions made in the deep nuclei of the cerebellum in several animals. This approach was supplemented by a second surgical procedure by utilizing a suction technique.

Morphological studies of the submammalian central nervous system. These anatomical experiments have proceeded along two major lines: (i) an analysis of the pigeon brain and (ii) the initiation of studies on the ascending spinal projections in the fish (the ray), amphibian (frog) and reptile (lizard) and of the efferent projections of the retina, optic tectum and cerebellum upon the brainstem in fish, amphibians and reptiles A stereotaxic atlas of the pigeon brain has been completed and is now being prepared for publication. The projections of the optic tectum of the pigeon have been studied and it was found that the tectum projects to the lateral pontine nucleus, the lateral mesencephalic reticular formation, nucleus cuneiformis, nucleus intercollicularis, central gray substance, nucleus isthmo-opticum and the stratum cellulare externum of the subthalamus. Ascending fibers were also traced to the nuclei dorsolateralis posterior thalami, pretectalis principalis and to other

pretectal nuclei. A significant finding is the rather prominent tecto-rotundal connection. This and other preliminary evidence on spinal and cerebellar afferent connections to the brainstem suggest that the tectum is the principal ascending pathway to the nucleus rotundus, and implies, furthermore, that earlier homologies of this nucleus with the ventrobasilar nuclei in mammals are erroneous.

Afferent peripheral nerve fibers to the brainstem and spinal cord. The termination of dorsal root fibers in the dorsal column nuclei and spinal gray matter were studied in the cat. Extradural lesions of the dorsal roots were made without laminectomy. Numerous fiber connections were traced to the nuclei gracilis, cuneatus medialis and cuneatus externus, and in the spinal cord to the nucleus proprius cornu dorsalis, the centrobasilar region of the dorsal horn and adjoining zona intermedia, to the nucleus cervicalis centralis and column of Clarke, and to a group of small cells ventrolateral to the central canal. Fewer degenerated fibers were traced to all ventral horn motor cells and small numbers of fibers were seen among cells in pars medialis cornu ventralis and the cells of the zona marginalis of Waldeyer in the dorsal horn. These findings were then compared with studies already completed on the corticospinal, tectospinal, rubrospinal, vestibulospinal and reticulospinal projections in the cat. Recently, this work has been extended to the rhesus monkey where a similar analysis will be conducted and the findings compared with those made in the cat.

Experimental-anatomical studies of the extrapyramidal motor system. The completed analysis of the striatofugal projection has been extended by an experimental study of projections from the substantia nigra. By a modified stereotaxic approach electrolytic lesions were placed in the substantia nigra of 6 cats and 1 monkey, the electrode entering the brainstem from the lateral side. By the use of the Nauta-Gygax silver technique for degenerating axons, fibers were traced to the thalamic nuclei ventralis medialis and ventralis anterior, to the superior colliculus, and to the midbrain tegmentum. Only few degenerating fibers could be followed to the globus pallidus and putamen, and none to the caudate nucleus. This latter negative finding is incompatible with the marked cell degeneration appearing in the nigra following large caudate nucleus lesions. No explanation for this discrepancy can be offered at this time. The fiber degenerations traced to the thalamus appear to furnish acceptable evidence of a hitherto unknown nigro-thalamic connection. Those traced to the superior colliculus and midbrain tegmentum cannot be interpreted reliably, as they could represent corticofugal connections interrupted in their passage through the substantia nigra. Further experiments in chronically hemidecorticated monkeys, now in progress, are expected to settle this question.

A histological study of retrograde axon degeneration. The fate of the central segment of severed axons appears to have received little attention, and it is commonly held that it does not differ materially from the rapid Wallerian disintegration shown by the peripheral (i.e. amputated) part of the axon. The present study was initiated 2 years

ago in an attempt to settle this question, the answer to which is of considerable importance for the interpretation of autoptical findings in case of chronic brain damage. The previous work on this subject has been continued in animals surviving high mesencephalic section of the medial lemniscus for periods of up to one year. Although the cell groups of origin of the medial lemniscus (contralateral nuclei gracilis and cuneatus) show heavy cell loss as a result of this surgery, the trajectory of the lemniscus proximal to the lesion shows no change other than a slow decrease of fiber caliber. A significant finding is that even after a 9 months survival massive Wallerian degeneration can be elicited in the atrophic bundle by lesion of the heavily "depopulated" nuclei gracilis and cuneatus from which it originates. The above-mentioned observations suggest that "retrograde fiber degeneration" is fundamentally different from the disintegrative process of anterograde (or Wallerian) degeneration. Unlike the latter, it is characterized by a slow fiber atrophy without drop-like disintegration of the fibers. The rate of atrophy in various stages of the process is currently being studied quantitatively by statistical sampling techniques.

A morphological study of the intrinsic organization of the spinal cord. The cellular structure of neurons in the spinal cord will be studied utilizing several variants of the Golgi method. Newborn animals are required for these studies as adult tissues appear largely refractory to the method. Camera lucida drawings will be made of different spinal cells and the dendrology and axonology of different cell types will be described. The findings will be compared with previous experimental results noted in the above paragraphs on afferent fibers to the spinal cord and of fibers arising from the brain and dorsal root ganglia. Presently, data are being collected on tissues taken from kittens and newborn pigs.

A quantitative analysis of neural tissue. Methods have been developed for accurately estimating the number of fibers or cells in the nervous system. Counts of the preganglion fibers to the superior cervical ganglion, and of cells in the superior cervical ganglion were made in cat, man, chimpanzee, rhesus monkey, spider monkey and baboon. These quantitative findings have been analyzed with statistical methods and new formulae derived for estimating the number of erroneously counted, fragments. It was found that the ratio of preganglionic fibers to ganglionic neurons varied from 1:28 in the squirrel monkey to 1: 196 in man. The number of cells, and the volume of the ganglion, increases with body weight. This increase in the size of the ganglion appears to be the result of an increase also in the average cell territory, or the average space occupied by each neuron. The average cell territory is reciprocally related to the number of cells per unit volume of tissue. This study, together with findings in the literature, have led to the formulation of a general theory that the average neuronal territory is a function of body size.

Histological techniques. A neurohistological stain has been developed which facilitates the recognition and measurement of nuclei and their

nucleoli. The essential ingredients are haematoxylin, neutral red and safranin O. The nucleoli and nuclear membranes stain a dark blue color while the Nissl substance stains a bright red. The background is very pale thus highlighting the cell body. This method can be modified so that the nucleolus alone is stained thus aiding in the possible future application of computer techniques in the estimation of cell numbers in different brain structures. Other technical experiments have resulted in the successful application of the Nauta silver method for degenerated axons to the central nervous system of fish, amphibians and reptiles. This is apparently the first time the method has been successfully used on the fish brain.

Experimental ammonia encephalopathy. Studies were initiated in order to test the hypothesis that ammonia is the responsible agent in human hepatic encephalopathy. Ammonia was infused into the cardiovascular system of rhesus monkeys, with the objective of inducing gliosis in the cortex and basal ganglia. The brains were removed after death and the tissues prepared according to histological methods: cresyl violet, haematoxylin and eosin, Heidenhain, PTAH, PAS, Cajal gold sublimate and Holzer's method.

Studies on the evolutionary development of sensory systems.

A) Visual pathways in birds. In birds the main pathway from the retina terminates in the optic tectum which is homologous with the mammalian superior colliculus. From the tectum fibers pass to the thalamic nucleus rotundus and from here projects to the forebrain, primarily to the ectostriatum. Pigeons were trained to perform brightness and pattern discriminations and lesions were made in the rectothalamic tract (connecting tectum and nucleus rotundus), nucleus rotundus, rotundo-ectostriatal tract and ectostriatum. All of these lesions resulted in severe deficits in visual discrimination performance. Following extensive post-operative retraining, the birds returned to the pre-operative performance levels. The effects of lesions on visual acuity, flicker and brightness difference thresholds, and color discrimination are also being studied.

B) Visual pathways in reptiles. A complementary study is in progress using the Tegu lizard. Once the animals have been adequately trained to make visual discriminations a similar series of lesions will be made and their performance tested.

C) Auditory Pathways in Birds. The auditory pathways of birds appear to be rather homologous with that of mammals. A seemingly important part of this system is the nucleus mesencephalicus lateralis, pars dorsalis (MLD) which appears to be the homologue of the mammalian inferior colliculus. This structure is extremely well developed in those avian forms, such as owls, which depend largely on acoustical cues for the tracking of prey. Pigeons are presently being trained to provide data on both pitch and loudness sensitivity. Lesions will be made in MLD and other related thalamic nuclei in order to determine the functional

role of these structures in audition.

Central nervous system control of autonomic reflexes.

A) Cardiovascular regulation in the bird. Cardiovascular reflexes are highly developed and extremely potent in birds, and, thus, offer a unique opportunity to study their inherent mechanisms. One such reflex is the diving reflex of many water birds. This reflex is being studied in acute preparation of wild and domestic ducks. An effort is being made to separate out the peripheral (trigeminal nerve) and the central (cortical and subcortical) inputs which initiate the marked vagal response seen during diving.

B) Viscero-sensory influences on cardiovascular reflexes. A major source of sensory input to the central nervous system is from the viscera. The role that this input has in modifying cardiovascular reflexes under both normal conditions and states of increased viscero-sensory discharge is being studied. Such phenomena as the well known changes in blood pressure and heart rate following sudden rapid distension of the bladder and stomach or following the sudden evacuation after prolonged distension are being studied in acute dog preparations.

C) Transducer development, data acquisition and computer techniques in the study of central nervous system regulation of blood flow.

1. Technical aspects of the system. Cardiovascular fluid dynamics are being studied by direct and indirect transducer techniques. Pulse volume signals obtained with fiber optic techniques, EEG signals, oxygen tensions, EKG, venous and arterial pressures are being recorded by techniques previously reported. Amplified signals are also being recorded on analog magnetic tape for subsequent waveform analysis. Signals of these variables are being digitized, fed into a format generator and recorded in digital magnetic tape. A Fortran program has been written for the IBM 1410 Digital Computer for printout of the one minute means and standard deviations of the heart period, pulse propagation time, systolic, diastolic, and venous pressures after systolic/diastolic relationships have been examined. Additional data acquisition refinement permits the preparation of raw data information. This includes a high speed multiplexer, A/D converter (ADCOMP), format generator, and digital tape drive. The multiplexer has 32 channels with a capability of sampling at 250,000 samples/second. Electronics permit random and sequential access to the data. The A/D converter samples and holds within 200 nanoseconds a ten-bit character at a maximum rate of 100,000 KC. It converts to a standard magnetic tape format at 800 frames per inch. This permits more complex statistical operations on maxima and minima, and inflection points. Spectral analyses of the data and more complex statistics are to be performed by a high speed digital computer currently available and being used on this problem. A three channel scanner was designed and fabricated to accept three pulsatile signals on a sequential scan in order to record more parameters of data digitally. Stability and reliability now achieve a 3% level. A Fortran II program has been

written for more complex statistical operations of data reflecting the cardiovascular and central nervous systems' behavior and their interrelationships. A program computing the correlation between systolic and diastolic pressure has been written. It is designed to test each variable for significant change using Student's t-test. The program is being refined to be applied to additional variables.

2. Application to human studies. Direct and indirect techniques for studying the cardiovascular fluid dynamics are being used to study relationships between the cerebral and peripheral vascular systems. Patients with disease in one or both carotid systems with known perfusion abnormalities are being compared with studies in which no disease was found. Analysis of wave components of the supraorbital arterial pulse obtained through the skin reflects certain cerebral perfusion changes as obtained from patient studies. Hydrogenated saline and ascorbic acid as tracer substances have proved to be useful in measuring blood flow velocity and cardiac output. These tracer substances are sensed at a catheter tip platinum electrode through oxidation-reduction potential changes. Through detection with a combined pressure sensor and accelerometer transducer (paleoencephalography) significant waveforms are being detected which correlate with the presence and absence of intracranial disease.

3. Application in animal studies. A study is in progress to examine many of above mentioned parameters in chronic monkeys during slow blood loss in an attempt to determine the central regulating mechanisms of blood pressure, heart rate and regional blood flow. These monkeys are studied continuously during control periods, during slow blood loss and during reinfusion periods. Preliminary evidence points to the following findings: a) Retransfusion of blood via the arterial as opposed to the venous side is more effective in maintaining life after shocking. b) A "plateau effect" has been noted, where the systolic and diastolic pressures (though not entirely simultaneously) decrease not steadily, but in a definite sequence of slopes and plateaus. c) A "second wind" phenonemon has been observed, where a blood pressure reduced to a very low level (eg 60 mm Hg) will rise to a higher level (eg 100 mm Hg) for 3-6 minutes even though exsanguination continues. d) An increased variability in all indices (systolic and diastolic pressures, heart rate, pulse propagation time), noted as an increased standard deviation in our representations, has been found to occur when blood pressure falls to its lowest ebb and also just before it does so. This may be an early indicator useful in determining pre-terminal shock. e) Direction is now toward varying oxygen and carbon dioxide administration to the animal at inflection points of blood pressure to alter their characteristic pattern.

Neuronal Coding in the CNS.

A) Response characteristics of units in the optic tectum. Discharge patterns of single cells are being studied in the optic tectum of the pigeon. Using microelectrode techniques the discharge patterns

are being related to different parameters of light stimulation. Duration of light flash, intensity and spectral components are being varied in an effort to characterize cellular response. The location of responding cells in both rostro-caudal and medial-lateral directions as well as the depth within the tectum is being studied using the varied stimuli. Preliminary data suggests that cells in the superficial layer of the tectum respond to light onset and cessation and perhaps react differentially to color. Cells lying in deeper layers appear to require more complex forms of light stimulations for activation.

B) Response characteristics of bladder receptors. Discharge patterns of single afferent fibers from the bladder are being studied in the cat. Utilizing micro-desection techniques small fiber fascicles are separated until responses are observed from single or few afferent fibers. Stimulation of the bladder either by filling with urine or spontaneous or evoked contractions is used to evoke receptor activity. Detailed statistical analysis of receptor discharge is then performed by a small computer designed and constructed in the division.

C) Response characteristics of single neurons to conditioned visual stimuli. The localization and discharge patterns of single neurons responding to natural stimuli have been extensively investigated. This study is an attempt to show how localization and discharge patterns of such single neurons are changed by properties of the stimuli other than modality, submodality and intensity. This study is being performed on chronic monkeys using micro-electrode techniques. Of special concern is the significance of the stimuli to the monkey. The monkeys are presented with physically similar visual stimuli of which some are signals for food; others are not. The monkeys having been trained to press a lever-operated feeding mechanism respond appropriately to the positive stimuli. An attempt is being made to discover whether these complex stimuli of significance to the animal will activate neurons which do not respond to a flash of light alone. The putamen, caudate nucleus and the lateral geniculate nucleus are being studied.

Effects of sleep and wakefulness on evoked responses following stimuli of psychological significance. Studies of the influence of sleep and wakefulness on evoked responses following click stimuli were extended. Evoked responses were studied in chronically implanted cats and averaged from the spontaneous EEG using a Mnemotron computer. Particular attention was paid to the psychological significance of the click stimulus and it was studied under conditions in which it was novel, habituated, conditioned and extinguished. In the early stages of click novelty and conditioning, sleep tended to increase the amplitude and complexity of the evoked response when compared to that observed in the awake animal. In later stages of conditioning, however, the usual differences between the novel and conditioned response were not maintained. Also, the diminution of response amplitude and complexity so characteristic of habituation and extinction were not maintained in the sleeping state.

Intracranial self-stimulation.

A) An experimental analysis of the positive and negative reinforcing properties of intracranial stimulation (ICS).

1. Earlier findings of this and other laboratories have indicated that the rate at which ICS is delivered is an important variable in determining both the sign and the strength of the reinforcing properties of electrical brain stimulation. This study is designed to investigate the aversive properties of ICS in an experimental situation in which the rate of stimulation is directly under the experimenter's control and not confounded by Ss responding. Rats, with electrodes implanted throughout the hypothalamus, have been tested in an escape paradigm. The experimenter initiates ICS at a given intensity and at a given rate. A lever press by S terminates the ICS for a fixed interval of time. The time from the initiation of stimulation until its termination by S is the response measure. All subjects tested have shown this escape behavior. A highly consistent and systematic relationship between intensity, train duration, off-time of stimulation and latency of termination has been found. Of special interest is the finding that some of the same subjects demonstrating this escape from ICS will work to initiate it at the same intensities, train durations and average rates at which they terminate ICS in the escape situation.

2. Related to the escape experiment is one in which the experimental conditions determine the motivational signs of the ICS reinforcement: positive, negative, or neutral. Rats are deprived of food to 80% ad and trained to lever-press for a food reward. In the test situation two levers are present. Pressing either lever results in the delivery of food, but only one results in ICS in addition. Periodically the other lever delivers ICS. Subjects so tested fall into one of three groups. a) Positive subjects that continue to respond following the lever that delivers ICS and food. b) Negative subjects who reliably avoid the lever delivering ICS after one or two brain stimulations. c) Neutral subjects who continue to respond on one lever at the same rate whether that lever produced food and ICS or food alone. By manipulating the parameters of current intensity it has been possible to change both positive and negative subjects to subjects apparently neutral. Other possible manipulations of subject response are under study.

3. An additional study is an attempt to determine if an ICS at a lower intensity than that needed to maintain self-stimulation can be used by the subject as a discriminable stimulus (S^d) on which to base a response. There is some reason to believe that at low current intensities the rate of delivery of the ICS and the stimulus train duration may interact to determine the duration ICS must be delivered before it can function as a S^d.

B) Analgesic effects of ICS. Recent experiments have suggested that ICS may have analgesic effects. This study is an attempt to corroborate this finding and, also, to quantitate the degree of analgesia

achieved. Rats, implanted with hypothalamic bipolar electrodes, have been tested for self stimulation behavior and rate intensity functions determined. Next, the animals are trained to escape foot shock and the shock escape threshold is determined using the method of constant stimuli. This threshold can subsequently be obtained during conditions of ICS at various intensities.

C) Discriminative conditioned emotional suppression during ICS. It has been shown that the normal suppressing CER fails to occur when positive brain stimulation in the septum is substituted for water reward. It might be concluded from this that normal auditory discriminative processes are inhibited in self-stimulation animals or that positive brain stimulation in some way helps to minimize the anxiety level produced by the conditioned stimulus. These alternatives are being tested by presenting the animal with 6 separate clicker frequencies in a random fashion during which the animal can obtain brain stimulation with every lever response. One of the click frequencies (CS) is always followed by an aversive foot shock. Rats with posterior hypothalamic placements show a marked suppression in their lever responding during the CS and a sharp generalization gradient in the responses to the other click periods. Other rats with septum and tegmental placements are also being tested.

D) Impedance changes during ICS. To evaluate the changes in impedance of brain tissue at the site of electrical stimulation, bipolar electrodes were implanted in the brain of monkeys, dogs, and rats. Impedance measures between the two electrode tips were made for various conditions of electrical stimulation. Impedance was initially high (about 100K ohms) before stimulation and declined to 10-20K ohms with repeated stimulation. The intensity and frequency of stimulation contributed to the speed of reduction of brain impedance. With frequency of stimulation higher than one stimulus train per day the impedance remained at the lower value.

E) Electrial self-stimulation in the region of the caudate nucleus in monkeys. Although monkeys perform inconsistently when arranged for intracranial self-stimulation such studies in monkeys are of interest because precise delineation of "rewarding" brain regions can be made only in relatively large brains. To determine whether or not stimulation of the caudate nucleus is reinforcing is of interest mainly for two reasons: a) In contrast to other "rewarding" brain regions (limbic lobe and hypothalamus) the caudate nucleus does not have a function related to basic reinforcers such as food, water, and sexual activity. If, therefore, caudate nucleus stimulation is rewarding the nature of the reward may be unrelated to the basic drives. b) In contrast to other "rewarding" brain regions the caudate nucleus does not have a high concentration of catechol amines. If the caudate nucleus is a "rewarding" region this falsifies a prevalent hypothesis that "rewarding" brain regions are coextensive with regions of high catechol amine content.

A method has been worked out where different brain sites are stimulated using different lead pairs of multilead electrode. Reproducible

brain stimulation behavior is obtained from the monkeys using an operant conditioning procedure involving a chained schedule. It seems that caudate stimulation is reinforcing but the monkeys are still alive and the placement of the electrodes unchecked.

It will be determined whether more intense brain stimuli are more reinforcing because the current spreads to a larger volume of tissue. If that is the case a monkey should selfstimulate as consistently as a rat when a comparable relative volume of the brain is stimulated.

Effects of brain stimulation on endocrine control. Previous work in this and other laboratories has shown direct effects of electrical brain stimulation on 17-OH steroid levels. An extension of this work was undertaken in order to define more precisely the parameters and sites of stimulation and relate them to the direction and duration of the effects. Stimulating electrodes were implanted in the hypothalamus, amygdala and hippocampus of chronic monkeys and plasma 17-OH steroids, BEI, glucose and insulin levels were determined. Single stimuli given every 5 or 10 seconds for 2 hours evoked no changes in the measured hormones although evoked potentials were recorded in other limbic areas.

Clinical studies in echoencephalography. Evaluation of A-mode echo-encephalography has continued in cooperation with the Neurosurgical service of Walter Reed General Hospital. The value of the technique in the detection and management of intracranial hemorrhage was presented to the Symposium Neuroradiologicum VII at New York City in September 1964, in a paper entitled "Echoencephalography As An Aid In The Evaluation Of Intracranial Hemorrhage" Mitchell J. Dreese, George J. Hayes, Ludwig G. Kempe and Lawrence McHenry. This article scheduled for publication in Acta Radiologcia stressed the value of serial readings and identification of echoes in addition to those from the cerebral midline.

Since the last report, B-scan equipment has also been acquired. Initial assessment suggests both advantages and limitations of B-scan technique compared to A-mode. Study of the clinical applications of both techniques is continuing in hopes of increasing the yield of data.

Technical developments.

1) Ultrasonic activity detector which provides a detailed measure of small animal activity within an enclosure. 2) Photo electric activity detector which measures the activity of larger animals in a cage. 3) Current-regulated biphasic stimulator used for precise electrical stimulation. 4) Stimulus isolation and current regulator units are a modification of the biphasic stimulator. 5) Sine wave stimulator is a battery-operated device automatically programed for zero average current flow. 6) Histogram analyzer; several types of on-line data processors were designed and built including, a) an inexpensive slow speed device using electro-mechanical components as well as electronic components. b) a higher speed instrument using a magneto-restrictive delay line as the memory element. 7) Cardiac Event analyzer automatically averages such

events as diastolic and systolic pressures, pulse pressure and heart rate over preselected periods of time and prints out tabulated data. 8) Electronic Pantograph is designed to plot on 11" by 17" paper the relative positions of single cells as viewed under a miscroscope. The exceptional gain and resolution of this system allows the plotting of points as close as lu. 9) Oxygen microelectrodes an improved electrode has been developed from platinum indium wire coated with glass. 10) Fiber-optic plethysmograph a major improvement has been made in the earlier models to allow adjustment of the system so that good contact with the cortex can be maintained. 11) Arterial and Venus catheters for chronic animals polyvinyl chloride has proven to be the best material so far studied. Prior soaking in heparin and saline help to maintain patency. 12) Cardiac Strain gauge has been developed for use in chronic experiments.

Summary and Conclusions:

Previous morphological and physiological studies of neural mediating mechanisms were extended. Several new projects were initiated. Morphological analyses of the mammalian, avain, reptilian and amphibian CNS was directed mainly toward the delineation of longitudinal systems interrelating successive levels of the central nervous organization. Physiological studies covered a wide range of neural mechanisms: A) peripheral and central control of autonomic reflexes; B) coding mechanisms of single neurons to light, mechanical, and complex behavioral stimuli; C) differential spontaneous and evoked activities of central nervous structures during sleep and wakefulness; D) neural mechanisms involved in intracranial self-stimulation; E) neural mechanisms involved in hormone release. Combined psycho-anatomical studies have been directed towards establishing functional properties of neuronal aggregates and their relationship to behavior. Studies with direct clinical applications were conducted using improved methods for measuring quantitative changes in cardiovascular parameters in normal and diseased patients and for echoencephalographic detection of pathological changes in cranio-cerebral topography. Several of the listed projects were accompanied and facilitated by new developments in technical instrumentation.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
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12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
016200 Stress physiol; 013400 Psychol (ind & group behavior); 005900 Environmental biol	08 63	NA	OTHER / DA

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19. GOV'T LAB/INSTALLATION/ACTIVITY
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TEL: 202-576-3559 TYPE: DA

21. TECHNOLOGY UTILIZATION: Medical illness

22. COORDINATION: NA

23. KEYWORDS: Stress; emotions; homeostasis; psychophysiology; neuroendocrinology; psychoendocrinology

24. (U) Tech Objective - Principal objective is to study central integrating mechanisms which control and coordinate visceral and metabolic functions. Understanding such mechanisms is essential to the understanding of bodily reactions to environment and stress, both psychological and physical, and of basic concern as an objective approach in psychosomatic medicine.

25. (U) Approach - This involves measurement of plasma and urinary hormone levels in monkeys and humans in a variety of acute and chronic stress situations, with emphasis on the concept developed by our earlier works that we must view changes in broad, overall hormonal patterns or balance, rather than in single endocrine systems as was previously customary in the stress field. Our major practical problems relate to the need for technicians to make multiple hormone assays prerequisite to hormone pattern work.

26. (U) Progress (Jul 64 - Jun 65) - A major part of recent effort in this program has been directed to the study of stress-related changes in hormonal balance prior to the onset of respiratory infections in Fort Dix recruits. Since this project involves collaboration with the Department of Virus Diseases, Division of Communicable Disease and Immunology, an integrated summary of the overall study is presented in a separate report. (See DA 0A6495, Code #6113011 3A013001A91C 01 113, Effects of physiological and psychological stress upon infection and disease.)

For technical reports, see Walter Reed Army Institute of Research Annual Progress Report, 1 July 1964 - 30 June 1965.

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DD FORM 1 AUG 64 1498 (Items 1 to 26 identical to NASA Form 1122) REPLACES DD FORMS 613 & 613C WHICH ARE OBSOLETE.

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 08, Physiology

Work Unit 077, Analysis of behavior and of mediating mechanisms: Neuroendocrinological factors

Investigators.
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Description.

This program is concerned with the measurement of regulatory changes in hormonal balance under the influence of psychological, physical, physiological and neural factors in both monkey and human subjects. Psychological stress experiments include studies of hormonal balance in acute and chronic conditioned emotional responses in monkeys and in natural life stress situations, such as the basic training cycle, in the human subject. Physical stress experiments include studies of hormonal balance in relation to trauma, cold, infection, food deprivation and posture. This year marked the initiation of neuroendocrine studies of medical illnesses, with investigation of stress-related changes in hormonal balance prior to the onset of respiratory infections in recruits at Fort Dix. Work on the development or improvement of hormone assay procedures as well as basic neuroendocrine physiological studies necessary for the interpretation of stress experiments have continued.

Progress.

Hormonal Balance in Psychological Stress

Considerable effort has been devoted to the preparation of a book covering a large series of experiments on the patterns of hormonal change (17-hydroxycorticosteroids, epinephrine, norepinephrine, thyroxine, estrogens, androgens, aldosterone, and insulin) occurring during and following conditioned emotional stress in the monkey. Laboratory work on this problem has been limited this year primarily to additional plasma insulin measurements during avoidance, which support our preliminary findings of decreased insulin levels during acute psychological stress in the monkey. Work on the series of manuscripts describing these studies and the revision of concepts which they introduce into the field of stress research is now about three-fourths completed.

Hormonal Balance in Physical Stress

Trauma. The series of patients undergoing surgical repair of ruptured intervertebral discs in which hormonal patterns are being studied has now been extended to 14 subjects. The findings in recent patients suggest that much of the variability of 17-OH-CS response to surgery noted in the first six patients was probably related to urine sampling problems. The last eight patients have all shown twofold or greater elevations in 17-OH-CS response, with a relatively small range of individual difference, so that attempts to correlate hormone response differences with psychological factors related to pain did not appear feasible. Recent availability of an improved urinary androgen method will soon permit completion of hormone pattern studies in these patients, which closely resembles the "psychological stress pattern" up to this point.

Cold. Attempts to study hormonal effects of sudden drops in environmental temperature in the monkey have repeatedly yielded evidence of concomitant psychological responses which make it difficult to evaluate changes related to cold per se. Because of our intensified interest in temperature effects on hormone levels in relation to the Fort Dix study of respiratory infections, two new approaches to this problem are currently under study. First, in the monkey we have designed a schedule of gradual lowering of environmental temperature over a period of 14 days in order to minimize the monkey's psychological reaction to temperature change. Secondly, we plan to study a platoon of basic trainees at Fort Dix during July under conditions as similar as possible to those of the platoon studied in February, except for the outdoor temperature. Comparison of the chronic mean hormonal patterns of the February versus July groups should yield information on changes related to environmental temperature extremes.

Food Deprivation. Because of the high probability that nutritional factors play an important role in the determination of chronic hormonal balance, a series of experiments have just been initiated in which the intake of carbohydrate, fat, protein, and salt are drastically varied one at a time. A basic complete control diet with weekly flavor changes including banana, orange, apple, and lemon is given for at least one month initially and between each diet change. One diet, flavored but without any nutritional value, is expected to provide a means of studying fasting without the psychological effects of an empty GI tract in the animal.

Posture. Because of the suggestion in our data that postural effects incident to hospitalization may have had a significant effect on hormonal balance in soldiers with respiratory infections, it is considered important to obtain systematic data on this problem. Basal hormone patterns have been determined on two monkeys housed in regular monkey cages inside a private cubicle for three months. These animals were then placed in experimental restraining chairs which maintain them

in a constantly upright posture. It is known that psychological adaptation to this situation normally occurs in three weeks or less, so that changes in hormone balance persisting beyond t s point may be related to positional or activity factors, the latter ch can be controlled experimentally.

Corticosteroid analyses are largely comp ted on most of the above physical stress studies, but the analyses of t remainder of the hormone patterns will be delayed for several months be use of the high priority given to the study of the pre-illness period in the Fort Dix subjects, described in the following section.

Hormonal Balance in Medical Illnesses

A major part of recent effort in this program has been directed to the study of stress-related changes in hormonal balance prior to the onset of respiratory infections in Fort Dix recruits. Since this project involves collaboration with the Department of Virus Diseases, Division of Communicable Disease and Immunology, an integrated summary of the overall study is presented in a separate report. (See Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH; Task 01; Work Unit 113, Effects of physiological and psychological stress upon infection and disease.)

Biochemical Methodology

A continuing effort to increase our volume of hormone analyses by the improvement and simplification of the present tedious hormone assay methods has resulted in the past year in a gas-chromatographic method for urinary androgen measurements which has quadrupled our output from 5 to 20 analyses per week. A double isotope-derivative method for measurement of urinary testosterone levels has also been developed and evaluated and is currently being applied to stress studies. Work is continuing on a radioimmunochemical method for plasma growth hormone method, but has been hindered by the difficulty in obtaining highly purified monkey and human growth hormone in sufficient quantities for preparation of antisera.

Physiological Studies

In order to evaluate the role of humoral influences on hormone response patterns, hormonal infusion experiments have continued. It has been found that epinephrine infused in relatively small amounts brings about a marked lowering of plasma insulin levels and greatly reduces the normal insulin response to injection of a large dose of glucose.

Summary and Conclusions.

This program is concerned with the neuroendocrine integration and coordination of physiological and metabolic adjustments associated with

stress. Increasing information is now accumulating that these integrating mechanisms operate, not by altering the secretion of one hormone at a time, but rather by effecting patterned changes in the overall hormonal balance in accordance apparently with the antagonisms and synergisms of the various hormones in their metabolic actions. Two major directions of work appear most urgent at present. The first concerns definition of the hormonal patterns associated with various physical factors such as cold, heat, diet, hypoxia, posture, exercise, and so on. The second concerns the study of disordered hormonal patterns as a possible pathogenetic factor in certain medical illnesses which often develop in a setting of sustained stress.

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