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WRAIR
ANNUAL PROGRESS REPORT

1 July 1967 - 30 June 1968

Volume I

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 1967 - 30 June 1968

Volume I

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 the 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."

TABLE OF CONTENTS

Volume I

PROJECT 3A013001A91C
IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01
In-House Laboratory Independent Research

RESEARCH AND TECHNOLOGY RESUME

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

DATE OF SUMMARY: 01 07 68 | KIND OF SUMMARY: D. CHANGE | 01 07 67 | SECURITY: U U | REGRADING: NA | RELEASE LIMITATION: OA | LEVEL OF SUM: A. WORK UNIT

CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 035

PRIOR NUMBER/CODE:

TITLE: (U) BIOCHEMICAL ACTION OF TRACE SUBSTANCES-EFFECTS OF TRACE METALS ON HORMONE AND ENZYME ACTIVITY

SCIENTIFIC OR TECH AREA: 002300 BIOCHEMISTRY 012900 PHYSIOLOGY

START DATE: 05 63 | EST. COMPL. DATE: NA | FUNDING AGENCY: OTHER DA

PROCUREMENT METHOD: C. IN-HOUSE

CONTRACT/GRANT: NA | DATE: | NUMBER: NA | TYPE: NA | AMOUNT: NA

RESOURCES EST.	PROFESSIONAL MAN-YEARS	FUNDS (IN THOUSANDS)
PRIOR 68	2	95
CURRENT 69	2	90

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21. TECHNOLOGY UTILIZATION: FOOD TECHNOLOGY

22. COORDINATION: NA

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 .

(U) APPROACH- ASPECTS OF GLUCOSE, PROTEIN AND FAT METABOLISM ARE INVESTIGATED IN CHROMIUM-DEFICIENT RATS AND IN DIABETIC PATIENTS, IN COLLABORATION WITH SCHROEDER, DARTMOUTH, AND WITH 3 OTHER 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.

(U) PROGRESS - JUL 67 THRU JUN 68 BIOCHEMICAL NATURE OF AN EYE LESION, PRODUCED BY COMBINED CHROMIUM PROTEIN DEFICIENCY WAS STUDIED. CORNEAS FROM LOW-CHROMIUM RATS BEHAVE SIMILAR TO THOSE FROM CHROMIUM-SUPPLEMENTED ANIMALS WHEN GLUCOSE METABOLISM AND RESPONSE TO INSULIN WERE MEASURED. AMINO ACID TRANSPORT WAS DEPRESSED IN THE CHROMIUM-DEFICIENT CORNEAS. CHROMIUM WAS NOT SIGNIFICANTLY TRANSPORTED INTO RAT EMBRYOES WHEN SIMPLE, INORGANIC SALTS WERE ADMINISTERED TO MOTHERS. ALL LITTERS EXAMINED CONTAINED NORMAL CONCENTRATIONS OF CHROMIUM, THEREFORE, CHROMIUM MUST HAVE BEEN TRANSPORTED ACROSS THE PLACENTA AS A SPECIAL DIETARY COMPONENT. ADMINISTRATION OF 51CHROMIUM-LABELED YEAST EXTRACTS GAVE A SIGNIFICANT RISE IN EMBRYOES, THEREFORE, THE ELEMENT MUST BE PRESENT IN A SPECIAL FORM (GLUCOSE TOLERANCE FACTOR)(GTF), TO BE FULLY AVAILABLE. PURIFICATION OF GTF ACTIVITY FROM BREWERS YEAST WAS CONTINUED. BIOLOGICAL ACTIVITY PARALLELED CHROMIUM CONTENT. GTF FRACTIONS POTENTIATE THE ACTION OF INSULIN AND ARE NOT A SIMPLE ADDITIVE EFFECT. OXIDATIVE PATHWAYS ARE STIMULATED MORE THAN ANAEROBIC PROCESSES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL REPORT, 1 JULY 1967 - 30 JUNE 1968.

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

31. MISSION OBJECTIVE: NA | 32. PARTICIPATION: NA

33. REQUESTING AGENCY | 34. SPECIAL EQUIPMENT

35. EST FUNDS (In thousands) | 36.

DD FORM 1 JAN 68 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

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, M.D.
Associate: CPT Duane E. Thurman
Edward E. Roginski, GS-11

Description.

The object of this research program includes the following goals: To define the mode of action of chromium in metabolism; establish the mode of interaction with insulin and related substances; determine metabolic and anatomic defects resulting from chromium deficiency; study the long-term effects of chronic chromium deficiency in animals and man and to correct existing deficiencies by adequate supplementation; define the organic, active complex in which chromium is present in biological systems; study its effect in various in vivo and in vitro systems; devise methods for large-scale production of sufficiently purified preparations for testing in patients; identify the active principle.

Progress.

1. Laboratory research. The emphasis in the laboratory was shifted from the study of the effects of simple chromium complexes to that of the naturally occurring chromium compounds. This shift was based on two reasons: The study of simple chromium salts depends to a large degree on the production of a chromium-deficiency, which, in turn, can be achieved only with a rigidly controlled diet making facility. The commercially available diets do not yet meet the standards of our previous in-house diet making facility. The naturally occurring chromium complex(es) has a much stronger effect, which can be demonstrated in systems that are only marginally chromium-deficient.

a. Corneal opacity as a symptom of chromium deficiency: This lesion is produced in 10-15% of rats raised under well-controlled low-chromium conditions. In an attempt to elucidate biochemical defects leading to the opacity, glucose and amino acid metabolism of isolated rat cornea was measured in vitro. Cornea did not respond to insulin (injected into the donor rat or added in vitro) with increased glucose oxidation. The effect of chromium (supplied in diet or added in vitro) was negligible; in contrast to other systems there was no potentiation of the action of insulin. The transport of α-amino isobutyric acid, a non-utilizable amino acid analogue,

was stimulated by insulin, given to the donor animal, and the magnitude of the insulin effect was significantly greater in the chromium supplemented than in the deficient rats. It remains to be shown whether these findings are also true for natural amino acids and whether they represent a major cause for the development of the lesion.

b. Studies on the effect of chromium on protein metabolism: The *in vivo* studies, showing that insulin promotes a greater utilization of some amino acids for protein synthesis in chromium-supplemented rats than in deficient controls, have been terminated and are written up for publication. Attempts to study this effect more closely in *in vitro* systems (homogenates, robosomal systems) produced unsatisfactory results and were postponed until a more consistent chromium deficiency can be produced.

c. Natural chromium complexes: Glucose tolerance factor (GTF).

(1) Chemical: The extraction procedure for the activity from Brewer's Yeast was improved and applied to a commercially available Brewer's Yeast concentrate with a consistently good yield of GTF activity. Chemical fractionation procedures were applied to these extracts, resulting in purified fractions of good biological activity. Batch procedures were developed to be applied prior to the expensive column filtration steps, in order to make the procedure applicable for pilot plant operation. These consist of adsorption of the activity on charcoal and selective elution with organic solvents. A proposal based on this method has been submitted to the Department of the Army, to initiate collaboration on a pilot plant scale with a commercial company.

Behavior of GTF activity under varied conditions of pH and heat was studied. Purified fractions from yeast grown in a ^{51}Cr-containing medium were further fractionated by gel filtration and their biological activity was compared to their chromium content. With the improved extraction procedure, biological activity was closely parallel to the ^{51}Cr content of the individual fractions.

(2) Biological: The following biological properties of GTF were established.

(a) GTF potentiates the action of insulin in chromium-deficient rats, as shown by a much steeper slope of the dose-response curve to insulin in the presence of GTF than in its absence.

(b) GTF is much more active than inorganic chromium complexes and it is less dependent on an optimal concentration.

(c) In epididymal adipose tissue, glucose oxidation is stimulated by GTF to a greater degree than is lipogenesis. In diaphragm, oxidation is increased by GTF, but the utilization of glucose for glycogen formation is unaffected.

(d) The utilization of pyruvate and other members of the glycolytic pathway is not affected by GTF.

(e) GTF acts qualitatively similar in systems measuring glucose uptake from the medium and in the manometric assay.

(f) Inorganic chromium salts, administered to pregnant rats, do not penetrate into the fetus. Repeated administration increases chromium transport only slightly. Chromium^{51} in form of glucose tolerance factor is easily transferred from mother into fetus. These data strongly indicate that chromium must be bound in a specific complex to exert maximal biological effects. The nature of this complex (GTF) is still unknown.

2. Collaborative outside studies:

a. A double blind study, conducted on prisoners in Wisconsin, failed to detect a beneficial effect of chromium supplementation for 16 weeks in 10 diabetics.

b. A collaborative study in Germany with 24 diabetics has terminated the first 18 cases. Of these, 9 responded to chromium supplementation with an improvement of glucose metabolism, in spite of an overall reduction of hypoglycemic medication; in 2 patients chromium was ineffective; in 7 there was a questionable effect.

Summary and Conclusions.

The studies reported here and the results of clinical studies suggest that an inorganic chromium salt is built into an organic complex (GTF) before it can exert its maximal biological effect. The failure of 50-65% of patients to respond to chromic chloride may be related to their inability to utilize chromium for the synthesis of GTF. The latter has a much stronger effect *in vivo* and *in vitro*. Its properties are more physiological, as evidenced by the ease with which it crosses the placenta and by its dose-response characteristics. GTF is chromium containing complex, present in Brewer's Yeast and other natural material. Its structure is still unknown.

Publications.

1. Mertz, W. The role of chromium in glucose metabolism. Proceedings 1st Annual Conference on Trace Substances in Environmental Health. Univ. of Missouri, 1967, p. 86.

2. Harlow, D. R., Mertz, W., and Mueller, J. F. Insulin-like activity from the sparganum of spirometra mansonoides. J. Parasitol., 53: 449 (1967).

3. Roginski, E. E., and Mertz, W. An eye lesion in rats fed low-chromium rations. J. Nutrition. 93: 249 (1967).

4. Mertz, W. Neue Gesichtspunkte zur Wirkung des Spurenelementes Chrom im, Kohlehydratstoffwechsel (German), Fortschr. Med., 85: 739 (1967).

5. Roginski, E. E., Feldman, F. J., and Mertz, W. Chromium in the newborn rat. Fed. Proc., 27: 482 (1968).

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6460	CSCRD-103

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

10. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 099

10B. PRIOR NUMBER/CODE:

11. TITLE: (U) CHROMIUM COMPLEXES OF INSULIN AND RELATED COMPOUNDS

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002300 INORGANIC CHEMIST 002300 BIOCHEMISTRY	06 63	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	06 68				
	b. NUMBER	DA 49 193 MD 2444	PRIOR FY 68	1	24
B. CONTRACT	c. TYPE	A-FPF d. AMOUNT $71,322	CURRENT FY 69	1	24

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
MEDICAL SCIENCE	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.

(U) APPROACH- THE INFLUENCE OF PH ON THE DEGREE OF OLATION AND OF VARIOUS POTENTIAL CHELATING AGENTS ON THE SOLUBILITY OF CHROMIUM IS DETERMINED USING A MEMBRANE DIALYSIS TECHNIQUE. NEW APPROACHES ARE IN PROCESS FOR SYNTHESIS OF NEW CHROMIUM COMPLEXES WITH LIGANDS OF BIOLOGICAL INTEREST, IN AQUEOUS AND NON-AQUEOUS SYSTEMS.

(U) PROGRESS - JUL 67 THRU JUN 68 PREVIOUS STUDIES MEASURING THE REACTION OF TRIVALENT CHROMIUM WITH BIOLOGICALLY IMPORTANT LIGANDS WERE EXTENDED USING EQUILIBRIUM DIALYSIS. IF SUCH LIGANDS ARE ABSENT, CHROMIUM WILL PRECIPITATE AT PHYSIOLOGICAL PH, DUE TO HYDROLYSIS AND OLATION OF THE AQUO COMPLEXES. MOST LIGANDS STABILIZE THE CHROMIUM COMPLEXES AS SHOWN BY INCREASED DIFFUSIBILITY OF THE RESULTING PRODUCT. THE INFLUENCE OF AGEING OF REACTION MIXTURES ON THE DIFFUSION PROCESS WAS STUDIED. ON THE BASIS OF THEIR ABILITY TO PREVENT AGE-INDUCED CHANGES, THE LIGANDS WERE PLACED IN ORDER OF THEIR RELATIVE COORDINATING TENDENCIES. IN DECREASING ORDER, THE MOST EFFECTIVE SUBSTANCES TESTED WERE CITRATE, TRIPHOSPHATE, PYROPHOSPHATE, SERINE, METHIONINE AND GLUCINE. SUGARS HAD LITTLE TENDENCY TO COORDINATE. FATTY ACIDS, SUCH AS OLEATE, TEND TO DECREASE THE SOLUBILITY OF CHROMIUM. THEIR EFFECT IS COUNTERACTED BY THE CHELATING AGENTS DESCRIBED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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.

DD FORM 1 JAN 65 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

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, Ph.D.
Associate: Walter Mertz, M.D.

Description.

To study the interaction of trivalent chromium with biological materials at physiological pH, to measure co-ordination reactions of chromium with potential ligands, such as amino acids, polypeptides, carbohydrates, and fatty acids, and to determine chemical parameters essential for biological activity of chromium complexes and to synthesize such complexes for biological testing.

Progress.

The biological role of chromium, principally enhancement of the action of insulin, depends on the chemical form of the chromium which is determined by competition for Cr(III) by various substances in the biological system. At physiological pH, the characteristic reaction of Cr(III) is olation (polymerization via bridging OH^- groups), and insoluble or high molecular weight products (assumed to be biologically unavailable) may also be formed by reaction of Cr(III) with other biological substances such as fatty acid ions. These effects may be more or less nullified or reversed by competitive co-ordinating agents forming low molecular weight rapidly diffusing Cr(III) compounds. These competitive reactions will establish the molecular weight range of the products and consequently the diffusibility of the Cr(III), which can be determined by dialysis.

In continuation of investigations described in previous reports, a dialysis procedure has been established by which biological substances can be screened with respect to their relative abilities to prevent formation of polymeric Cr(III) reaction products. The method is convenient, generally applicable to all the substances of interest under all relevant reaction conditions, and it is quantitative in the sense that the results are numbers establishing an order of stability of the reaction mixtures and consequently an order of relative tendencies of the biological substances tested to co-ordinate with Cr(III).

Dialysis curves are obtained showing fractional attainment of dialysis equilibrium (of Cr(III)) versus time. The relative area under the curve is proportional to the rate of diffusion and thus inversely proportional to the average molecular weight of the diffusing species. As the reaction mixture ages, the change in area under the curve is a sensitive indicator of the occurrence of reactions, in particular olation and other reactions leading to

increase in molecular weight of the diffusing species on aging of the reaction mixture. Thus, the smaller the change, the more stable the reaction mixture, i.e., the more effective is the compound under test in competing with hydroxide ion and other substances forming high molecular weight complexes with Cr(III).

By this procedure, the compounds of interest can be placed in an order of their relative stabilizing tendencies. Amino acids are more or less effective depending on their molecular weights and composition; the most effective of those tested are serine, methionine and glycine. The most effective of all substances tested are citrate, pyrophosphate and triphosphate. At physiological pH these substances in appropriate concentrations stabilize Cr(III) reaction mixtures for days; moreover, they nullify the effects of competing substances such as fatty acid ions.

Biological systems, of course, contain only trace concentrations of pyrophosphate and probably no free triphosphate. On the other hand, organic derivatives of these inorganic ions are abundant. Examination of their structure indicates that they can be expected to be effective complexing agents and investigation of these substances is therefore definitely planned.

Typical of the many results obtained are those of the following table, for reaction mixtures characterized as follows: Cr(III), 10^{-4} M; pH, 7.0; $PO_4^{\equiv}$; 0.02 M. The symbols used have the following meanings:

R' % attainment of dialysis equilibrium:

F' 100 x ratio of area under dialysis curve of reaction mixture to area under dialysis curve for $Cr(H_2O)_4Cl_2^+$, taken as standard of comparison:

P' 100 x ratio F' (aged reaction mixture)/F' (unaged reaction mixture).

Q' % decrease in area under dialysis curve caused by changes in composition due to aging of reaction mixture: Q' is inversely proportional to the stability of the reaction mixture and to the effectiveness of the co-ordinating agent.

The versatility of the procedure is apparent from these results which show effect of reagent, of aging time of reaction mixture, and of concentration of reagent. Many other results for which space is not available here show also the effect of pH and competition between, for example, the polyphosphates and fatty acid ions, and show also the reversal of olation by effective co-ordinating agents. In addition to the competition of various substances for Cr(III), there is the possibility of competition of Cr(III) with other metallic ions for the same ligand (a phenomenon which it is planned to investigate), e.g., Fe(II), Fe(III), Cu(II) and Zn(II).

It is anticipated that the procedure described can be made even more versatile by suitable modifications. Among the variations either under

investigation or being planned are the following: use of microdialyzers to permit economical investigation of expensive biological materials; investigation of membranes other than regenerated cellulose not only because of possible effects of different materials but also to study a range or pore diameters; utilization of an automatic continuous dialysis procedure in which the reaction mixture is pumped continuously through the dialyzer, and a counting tube in the well of the gamma scintillation detector connected to an automatically recording counter.

Reagent	Conc., M	Aging Time	R'	F'	P'	Q'
Pyrophosphate	10^{-2}	1 hr.	97.20	95.25	-	-
		24 hrs.	98.66	91.18	96.40	3.60
		1 wk.	99.31	94.12	98.81	1.19
		2 wks.	98.72	93.43	98.09	1.91
	10^{-3}	1 hr.	98.80	95.49	-	-
		24 hrs.	98.72	92.94	97.34	2.66
		1 wk.	96.28	88.98	93.18	6.82
	10^{-5}	1 hr.	92.00	84.37	-	-
		24 hrs.	82.63	70.63	83.72	16.28
Triphosphate	10^{-2}	1 hr.	99.64	96.66	-	-
		24 hrs.	97.51	94.09	97.36	2.65
		1 wk.	98.76	93.19	96.41	3.59
		3 wks.	100.00	95.16	98.45	1.55
	10^{-3}	1 hr.	99.87	95.19	-	-
		24 hrs.	98.31	92.34	96.99	3.01
		2 wks.	97.88	91.92	96.57	3.43
Citrate	10^{-2}	1 hr.	97.49	94.73	-	-
		24 hrs.	100.00	95.34	100.64	-0.64
		11 d.	100.00	94.25	99.48	-0.52
	10^{-3}	1 hr.	97.15	93.93	-	-
		24 hrs.	96.63	86.62	92.22	7.78
		1 wk.	97.97	86.31	91.89	8.11
Glycine	10^{-2}	1 hr.	93.84	87.78	-	-
		24 hrs.	86.59	76.10	86.69	13.31
Glucose	10^{-2}	1 hr.	90.41	84.16	-	-
		24 hrs.	74.77	61.42	72.98	27.02

Summary and Conclusions.

The principal biological role of chromium is an enhancement of the action of insulin. Establishing the mechanism of this effect requires a knowledge of the reactions of Cr(III) with biological substances at physiological pH. By means of dialysis, such substances can be readily tested to determine their relative abilities to keep Cr(III) in the form of low molecular weight rapidly diffusing species. Dialysis curves are obtained showing fractional attainment of dialysis equilibrium versus time. The area under the curve is proportional to the rate of diffusion, and therefore inversely proportional to the average molecular weight of the diffusing species. As the reaction mixture ages, the change in area under the curve is a sensitive indicator of the occurrence of reactions, in particular, olation and other reactions leading to increase in molecular weight of the diffusing species. Thus, the smaller the change the more stable the reaction mixture; i.e., the more effective the compound under test in competing with hydroxide ion and other substances forming high molecular weight complexes with Cr(III). On this basis, the compounds of interest can be placed in an order of their relative co-ordinating tendencies. Amino acids are found to be more or less effective depending on their molecular weights and composition; the most effective of those tested are serine, methionine and glycine. The most effective of all substances tested are citrate, triphosphate and pyrophosphate. At physiological pH these substances in appropriate concentrations stabilize Cr(III) reaction mixtures for days; moreover,they nullify the effects of competing substances such as oleate. (U)

Publication.

Rollinson, C. L., Rosenbloom, E., and Lindsay, J. Reactions of chromium(III) with biological substances. Proc. 7th Internatl. Congress Nutrition, Vol. 5, p. 692-697, 1967.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6467	CSCRD-103

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

10a. CURRENT NUMBER/CODE
61130011 3A013001A91C 00 105

10b. PRIOR NUMBER/CODE

11. TITLE (U) METALLIC MICRONUTRIENTS AND INTERMEDIARY METABOLISM

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

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	07 64			
	b. NUMBER DA 49 193 MC 2595	PRIOR FY 68	1	28
B. CONTRACT	c. TYPE A.FPF d. AMOUNT $81,584	CURRENT FY 69	1	28

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
FOOD TECHNOLOGY	NA

23. KEYWORDS
CHROMIUM, VANADIUM, NICKEL, GERMANIUM, MINERAL METABOLISM, ARSENIC, TIN, ZINC.

24.

(U) TECH OBJECTIVE - TO STUDY THE EFFECTS OF CHRONIC NUTRITIONAL DEFICIENCY OF METALLIC TRACE ELEMENTS IN ANIMALS, WITH EMPHASIS ON DETECTION OF LONG-TERM DEGENERATIVE PROCESSES. ELEMENTS INCLUDE CHROMIUM, VANADIUM, NICKEL, GERMANIUM, NIOBIUM, ZIRCONIUM, ARSENIC, ANTIMONY AND TIN.

(U) APPROACH- ANIMALS ARE RAISED IN A SPECIAL LABORATORY ALLOWING STRICT CONTROL OF TRACE METAL CONTAMINATION. DIETS, DEFICIENT IN A SELECTED ELEMENT, BUT SUPPLEMENTED WITH ALL OTHER ESSENTIAL DIETARY FACTORS, ARE FED ANIMALS DURING THEIR LIFE SPAN. CHEMICAL, PATHOLOGICAL, HISTOLOGICAL EXAMINATIONS AND FUNCTIONAL TESTS ARE MADE ON DEFICIENT ANIMAL GROUPS RECEIVING DEFICIENT, NORMAL, EXCESSIVE AND TOXIC AMOUNTS OF THE ELEMENT.

(U) PROGRESS - JUL 67 THRU 30 JUN 68. FOURTH-GENERATION RATS WITH LONG-TERM DEFICIENCY, EXHIBITED INCREASED LEVELS OF CIRCULATING CHOLESTEROL. CHROMIUM SUPPLEMENTATION (1 PPM) IN WATER RESTORED LEVELS OF CLC MALE ANIMALS TO NORMAL. FEMALE RATS REQUIRED 5 PPM. NIOBUM AND NICKEL LOWERED, AND TELLURIUM, SELENIUM AND ANTIMONY INCREASED CHOLESTEROL LEVELS. IN MAN THE ACUTE RISE OF PLASMA CHROMIUM BY ORAL GLUCOSE LOAD INCREASED URINE EXCRETION OF CHROMIUM 2-FOLD. MOLASSES, RAW AND REFINED SUGARS WERE ANALYZED FOR CHROMIUM. REFINEMENT LOSS EXCEEDED 90 PERCENT OF THE ORIGINAL RAW CONTENT. CADIUM INDUCED HYPERTENSION IN RATS BY LONG-TERM FEEDING AND ALSO BY INJECTION OF ONE DOSE. HYPERTENSION RETURNED TO NORMAL WITH ZINC-CHELATE TREATMENT. LIFE-TERM STUDIES WITH ARSENIC, GERMANIUM, TIN AND VANADIUM HAVE BEEN CONCLUDED. NONE OF THESE AFFECTED GROWTH. SELENATE AND TELLURITE (2 PPM IN DRINKING WATER) WERE WELL TOLERATED BY RATS. SELENITE HAD CONSIDERABLE TOXICITY. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Independent Laboratory Research

Work Unit 105, Metallic micronutrients and intermediary metabolism

Investigators.
Principal: Henr A. Schroeder, M.D.
Associate: Walter Mertz, M.D.
D. F. Frost, Ph.D.
A. P. Nason, B.S.
J. J. Balassa, Ph.D.

Description:

The general purpose of this research is to ascertain whether or not any common chronic diseases of industrialized man result from a) accumulation of a specific abnormal trace element, or b) from marginal deficiency of an essential trace element. To approach this problem, one needs to know whether or not tissue deficiency of an essential trace element occurs in man, especially as a function of aging, and which abnormal elements accumulate in tissues and which do not. The next step is to expose animals to each element and observe effects.

Small mammals with a feasibly short life span, mice and rats, are born and raised in an environment as free of airborne and other metallic contaminants as possible. An unique animal laboratory was specially built for this purpose. The diet of seed rye flour, corn oil and dry skim milk, fortified by vitamins, is low in most trace elements. The essential trace metals, zinc, copper, manganese, cobalt, molybdenum, and chromium are added to doubly deionized drinking water in amounts equivalent to their concentrations in a standard laboratory chow. To the basic drinking water is also added one abnormal trace element, usually at 5 ppm, as a soluble organic complex (acetate or citrate).

Weanling mice and rats, born in our laboratory, equally divided as to sex, numbering about 100, are given this drinking water for their lifetimes, which under these conditions have lasted up to 30 months for mice and 52 months for rats. Functions measured in mice are growth rates, weights, survival and longevity, microscopic pathology and incidence of tumors; these are measured in rats as well as urinary protein and glucose, blood pressure, serum cholesterol, glucose and uric acid, visual estimation of aortic plaques, and amounts of aortic lipids. In both types of animals, heart, lung, kidney, spleen, and liver are ashed and analyzed for the metal given. An equal number of controls are treated identically. Analyses are made by microchemical, fluorimetric and atomic absorption spectrophotometric methods.

Progress.

Effects of arsenic, germanium, tin, and vanadium in mice were evaluated. The addition of 1 ppm chromium(III) acetate improved the growth rates and survival curves of controls, increasing longevity. Tin accumulated in heart and spleen but was not toxic in terms of life spans and longevities. Germanium accumulated in spleen and decreased life span and longevity. Arsenic accumulated to some extent in liver, heart, and lung, decreasing life span and longevity. Growth rates were unaffected, but body weights of older animals were suppressed as follows: Arsenic in males, germanium and tin in both sexes. No significant effects of vanadium were found.

Effects of zirconium, niobium, antimony and fluorine (10 ppm) were evaluated in mice. Niobium and antimony were somewhat toxic in females, accumulated in tissues, and hepatic fatty degeneration occurred with niobium. Zirconium was relatively non-toxic and no accumulation was found. Fluoride was non-toxic in these terms.

Effects of germanium, tin, and arsenic were evaluated in rats. There were no effects on growth. Tin was toxic in terms of life span and longevity in females and germanium in both sexes, producing fatty degeneration of the liver. Renal tubular vacuolar degeneration also occurred with tin. Both elements accumulated in tissues, germanium only to slight extent. Arsenic was non-toxic in spite of large accumulations in all tissues, especially aorta and red blood cells.

Spontaneous tumors were suppressed in mice fed germanium and arsenic and in rats fed germanium. None of ten elements was tumorigenic or carciogenic when given by mouth.

The feeding of selenium as selenite but not as selenate caused marked early mortality in rats, but not in mice. Serum cholesterol levels were elevated in rats deficient in chromium and low in those fed chromium. Of 13 elements, low levels were found when fed niobium, chromium, and nickel, and high levels when fed tellurite. Differences between levels of males and females were found for germanium, cadmium, and tin, female values being higher, and for vanadium, chromium and selenium, female values being higher. Chromium, especially, appears to be anti-cholesterogenic in rats, and in some human beings.

Surveys of environmental sources and exposures, and tissue concentrations by age were published for tellurium, zinc and cadmium, germanium and cobalt, involving hundreds of analyses. The role of chromium in mammalian nutrition was reviewed. Human tissue concentrations of lead from U.S. and foreign subjects were compiled. Lead accumulates in American but not foreign tissues, except for aorta, where it accumulates in both groups.

Further experiments on cadmium hypertension in rats have shown that the mechanisms of this disorder and renal ischemic hypertension are dissimilar, although their effects are additive. Failures to induce hypertension by partial constriction of the renal artery were converted into successes by

feeding cadmium in drinking water for three weeks. Hypertension was rapidly induced by renal ischemia in the absence of renal cadmium, and cadmium hypertension was slowly induced (two years) in the absence of renal ischemia. The injection of a zinc loaded chelate, Na_2Zn CDTA, little affected renal ischemic hypertension but abolished cadmium hypertension, displacing renal cadmium with zinc in both cases. Renal ischemia resulted in a loss of zinc and copper, but not cadmium or chromium, from the ischemic kidney, increasing the ratio of cadmium to zinc. The chelate reversed this increase.

In acute experiments using direct measurements of blood pressure, rats fed cadmium for two months had higher mean pressures than did cadmium-free controls, although few were actually hypertensive. These rats showed lessened responses to the intravenous injection of norepinephine and angiotensin. This diminished response was restored to normal by injection of the zinc chelate. Intraperitoneal injection of cadmium was also followed by an immediate rise in blood pressure. This chelate of zinc has been evaluated as to toxicity and is ready for clinical trials.

The following 11 elements are being given to mice at 5 ppm in drinking water except as noted: scandium, gallium, selenite (3 ppm), selenate (3 ppm), molybdenum, rhodium, palladium, indium, tellurite (2 ppm), tellurate (2 ppm), chromium $^{3+}$ (as the potassium oxalate, 12 ppm). Administration of yttrium and chromium $^{6+}$ will begin shortly.

The following 11 elements are being given to rats at 5 ppm in drinking water except as noted: Vanadium, chromium (12 ppm), nickel, selenite (3 ppm), zirconium, niobium, molybdenum, cadmium, antimony, tellurite (2 ppm), lead.

Preliminary evaluation of data based on incomplete or nearly complete life spans has demonstrated toxicity for mice exhibited by selenite, tellurite, scandium, gallium and possibly indium. For rats, toxicity in one form or another has appeared for antimony, cadmium, tellurite and lead.

Attempts are being made to cause more severe forms of atherosclerosis than we have formerly produced by chromium deficiency. Using a diet of sugar, torula yeast, and lard, varying the type of sugar (raw, brown, white) and eliminating manganese, copper, or zinc from the basic drinking water, we are attempting to induce a conditioned deficiency of a trace element and evaluate its effects upon gross and microscopic alterations in blood vessels, aortic content of lipids, and changes in serum glucose and cholesterol levels. Preliminary analyses have disclosed lower fasting glucose levels in rats fed brown sugar than those fed white sugar.

Surveys of the environmental sources and exposures of human beings to each of 16 trace elements have been published, and to one bulk element, magnesium, has been completed. Surveys on selenium, antimony, and molybdenum have been begun--the methods are slow and cumbersome.

Analyses for chromium on all carbohydrate foods available and on a number of fats are completed. When major sources of calories are refined, chromium is partly lost to a large extent in sugars.

Six diabetic patients have been given 1.0 mg of chromium(III) as the chloride, acetate, or oxalate daily for the past two years. In one case glucose tolerance has returned to normal. The other five have been given, in addition, 10 mg manganese as the acetate daily for two months. Slight improvement in glucose tolerance has been found in three and marked improvement in one. It is too early to tell whether improvement will be continuous.

Summary and Conclusions.

In order to evaluate biological effects of trace elements, mice and rats were exposed for their lifetimes to small doses of each of many essential and abnormal elements in drinking water, in a laboratory and on a regimen designed to avoid environmental contamination. Growth rates, survival and longevity, microscopic pathology of tissues, concentrations of trace elements in tissues and in rats, blood pressure, serum cholesterol, glucose and uric acid, aortic plaques, and lipids and tumor rates were measured or examined. Surveys of the human environment for six elements in foods, water, vegetation, wild animals were also made by trace element analysis, and human tissue concentrations for four. Arsenic accumulated in mouse and greatly in rat tissues; it was slightly toxic only to mice. Germanium was somewhat toxic to mice and rats and depressed tumor rates, accumulating in tissue. Tin accumulated and was toxic to rats but not in mice. Vanadium was non-toxic to mice. Zirconium did not accumulate and was non-toxic; antimony was toxic to both species and accumulated. Niobium was slightly toxic to mice but not to rats. Chromium deficiency induced elevated cholesterol and glucose levels as did tellurite. Selenite was toxic, selenate not. A zinc chelate of CDTA abolished cadmium hypertension in rats and removed some renal and hepatic cadmium. Cadmium feeding or injection raised blood pressure and diminished responses to norepinephrine and angiotensin. Lead accumulated in American but not foreign human tissues with age. Normal dietary intakes of germanium, tellurium, zinc, cadmium and cobalt were ascertained. Preliminary conclusions from this and previous work are that renal cadmium is an accessory factor in human hypertension, its mechanism differing from that of renal ischemic hypertension, that chromium deficiency may be an accessory factor in atherosclerosis, that other trace elements may suppress spontaneous tumors in mice, and that a number of other trace elements are either inert or exert vague toxicity not associated with any demonstrable disease.

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RESEARCH AND TECHNOLOGY RESUME

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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.

(U) APPROACH- TO STUDY THE PHYSICOCHEMICAL CHARACTERISTICS OF ANTIBODY OF SERUM AND NASAL SECRETIONS USING GEL FILTRATION, ULTRACENTRIFUGATION, RADIOAUTOGRAPHY AND ELECTROPHORESIS TO DETERMINE SPECIFIC DIFFERENCES AND SEQUENTIAL APPEARANCE FOLLOWING IMMUNIZATION AND INFECTION IN ANIMALS AND MAN.

(U) PROGRESS - JUL 67 THRU JUN 68 ABERANT REACTIONS OF CHILDREN TO LIVING ATTENUATED MEASLES VACCINE HAVE BEEN STUDIED TO DETERMINE THE NATURE OF THE PATHOLOGICAL RESPONSE. CHILDREN RECEIVING LIVING VACCINE 1-2 YEARS AFTER INITIAL IMMUNIZATION WITH FORMALIZED RUBEOLA VIRUS DEVELOP IN 36-72 HRS AN ERYTHEMATOUS-HEMORRHAGIC REACTION WITH EDEMA AND SWELLING IN THE INOCULATION SITE. ON BIOPSY, THESE REACTIONS ARE CHARACTERIZED BY INTENSE NEUTROPHILIC INVASION, NECROSIS OF INTIMAL CELLS OF ARTERIOLES, AND CAPILLARY BLEEDING. IMMUNOFLUORESCENT STUDIES OF SMALL ARTERIAL VESSELS IN SUCH BIOPSIES REVEAL THE PRESENCE OF COMPLEMENT BINDING COMPLEXES OF MEASLES VIRUS AND GAMMA-G IMMUNOGLOBULIN. SUCH COMPLEXES ARE CHEMOTOXIC FOR LEUKOCYTES WHICH IN TURN DESTROY VASCULAR ENDOTHELIUM. THIS MAY BE THE BASIC PATHOGENETIC PROCESS FOR A NUMBER OF THE SO CALLED HYPERSENSITIVITY REACTIONS IN SEVERAL DIFFERENT VIRUS DISEASES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 106, Physico chemical studies of the biologic significance of antiviral antibody

Investigators.
Principal: Joseph A. Bellanti, MD
Associates: Edward L. Buescher, COL MC, Malcolm S. Artenstein, Barbara Klutinis, Brigette Cantz, Margaret Moffitt, Mamie Barr

Description.

This is a definitive study of the biologic significance of antiviral antibody in order to assess its role in immunity in man. Control mechanisms and sequence data are being sought. The study of serum and local respiratory antibody responses has been performed in children immunized with inactivated and attenuated measles virus vaccines. The data suggest that although the distribution of serum antibody is quite similar following the use of both vaccines, respiratory γA- associated antibody is produced regularly only after immunization with attenuated vaccine. In addition, studies of characterization of nasal and serum antibody responses in recruits infected with adenovirus type 4 have continued as well as the study of responses of children infected with Salmonella. These studies have included the use of radioimmunodiffusion techniques.

III Progress:

A. Physicochemical studies of humoral and local antibody responses to infection and immunization.

1. Respiratory viruses

a. Serum and local antibody responses in children following immunization with attenuated and inactivated measles vaccine

A major area of investigation of this laboratory over the past year has been a continuation of studies with measles virus vaccines reported in last year's annual report (1966-67).

Background

In recent years, a growing body of evidence has accumulated which suggests that the presence of antibody in local respiratory secretions, primarily of the IgA variety (Annual Reports 1964-65, 1965-66, 1966-67) may be a more important index of host resistance to certain viral infections than circulating antibody. Further, in volunteer studies, it was demonstrated that although injection of an inactivated type 1 parainfluenza virus vaccine produced serum antibody responses similar to those following upper respiratory tract infection with type 1 virus, immunization with inactivated vaccine was significantly less effective in stimulating the appearance of IgA antibody in nasal secretions. These observations suggested that the presence of serum antibody alone or its induction by vaccines may be inadequate to assess the effectiveness of certain viral vaccines.

Up until recently, two types of measles vaccines were available for immunization: an inactivated and a live attenuated vaccine. Several studies have indicated that both vaccines are capable of inducing good levels of serum antibody. However, the duration and degree of protection afforded by the inactivated vaccine appears to be inferior to that of the attenuated. More distressing than the apparent lack of clinical effectiveness, however, have been several recent reports of unexpected reactions in recipients of the inactivated vaccines who later receive live vaccine or who are exposed to natural measles.

The present studies were conducted in order to compare the ability of inactivated and attenuated measles vaccines to stimulate antibody in serum and nasal secretions of children and to study the immunochemical characteristics of such antibody. The possible application of these findings to the recently described problem of altered reactivity of the host to inactivated vaccine is currently under study.

Subjects: These studies were conducted at the Children's Convalescent Hospital, Washington, D.C. and at Georgetown University Medical Center, Department of Pediatrics. The study group consisted of 24 children whose ages ranged

from 10 months to $3\frac{1}{2}$ years and in whom a history of measles infection or measles immunization could not be obtained. Despite this, pre-bleedings revealed that 8 children, or 33% of the group had serological evidence of prior measles infection (Table I). Administration of inactivated and attenuated vaccines was assigned arbitrarily and resulted in the creation of four subgroups (Table I).

Vaccines. A formaldehyde - inactivated, alum-precipitated measles virus vaccine* was administered to 7 children whose pre-immunization sera lacked measles neutralizing antibody and to 5 children whose pre-immunization sera revealed the presence of antibody (Table I). The vaccine was administered in a series of 3 injections of 0.5 ml. each given at 0,1, and 2 months.

A live attenuated measles virus vaccine, Schwarz strain (Lirugen)**, was administered to 9 children whose pre-immunization sera lacked measles neutralizing antibody and to 3 additional children found to be seropositive (Table I).

Collection and processing of nasal secretions and sera. The methods used for the collection and processing of nasal secretions and sera were essentially those described previously (Annual Reports 1964-65), 1966-67).

Immunochemical Characterization of antibody. Protein concentrations of nasal secretions were determined by the Lowry Method, and immunoglobulin concentrations by the agar diffusion method. Selected sera were also fractionated by density gradient ultracentrifugation.

Radioimmunodiffusion studies. Because of limitations of the resolution of separation of immunoglobulins by physico-chemical techniques and of limitations in the amounts of nasal secretion specimens, a radioimmunodiffusion technique was developed for these studies based on the method described in last year's annual report for adenovirus.

Briefly, Edmonston strain of virus was grown in the presence of C^{14} uniformly labeled amino acids. Following 6 days of growth the cell sheets were washed and harvested into 15 ml Hank's balanced salt solution. The fluids were frozen and thawed five times to release virus and then centrifuged at 500 g per 15 minutes to remove cell debris and dialyzed against phosphate buffered saline. The preparation used for radioimmunodiffusion studies contained 340,000 cpm per ml.

Radioimmunodiffusion studies were performed on selected sera and nasal secretions using the indirect method described for adenovirus (Annual Report 1965-66).

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*Inactivated measles vaccine prepared in monkey kidney tissue culture, Chas. Pfizer & Co., Inc.

**Attenuated measles virus vaccine prepared in chick embryo tissue culture, Pitman-Moore Division of the Dow Chemical Company.

Immune response of seronegative children to immunization with inactivated vaccine. Six of seven children immunized with three monthly doses of inactivated vaccine, all of whom lacked neutralizing antibody in their pre-immunization sera, responded with serum neutralizing antibody (Fig. 1, Table II). Serum antibody was first detected by the 28th day in one child and in 6 of 7 children by 42 days. The serum antibody response followed a classical immunological response with increments of rise noted after each dose of inactivated vaccine (Fig. 1).

In contrast to the antibody response in serum, nasal antibody was detected in single specimens from only 2 children on the 56th and 70th days respectively. (Fig. 2, Table II). The nasal antibody titers in these specimens were 1:2 and 1:8 with corresponding serum antibody titers of 1:64 and 1:1024, respectively.

Immune response of seronegative children to immunization with attenuated vaccine. All seronegative children who received attenuated vaccine responded quite promptly with serum antibody following a single injection of attenuated vaccine (Fig 1, Table II). Serum antibody was detected in all specimens tested by the 14th day reaching maximum levels by 42 days. These levels were sustained for at least 84 days.

Nasal antibody was first detected by the 14th day in 3 of 8 specimens, reached a maximal titer in 5 of 8 specimens and remained detectable until 70 days (Fig 2, Table II). Nasal antibody was detected at 170 days in 1 of 3 followup specimens tested.

Immune response of seropositive children to immunization with either inactivated or attenuated vaccines. Eight children found to contain antibody in pre-immunization sera, presumably the result of natural infection, were also studied (Table I). Two of 3 children immunized with attenuated vaccine showed a two-fold or greater rise in serum antibody; of 5 receiving inactivated vaccine, 4 showed a four-fold rise.

Nasal antibody was detected in 5 of 8 seropositive children. In 7 of these 8 children a two fold or greater rise of nasal antibody was noted following immunization with either vaccine. Thus, it appears that nasal antibody is more regularly stimulated following the rise of attenuated vaccine, but once produced, it can be stimulated once again by either vaccine.

Characterization of Proteins and Antibody in Nasal Secretions. Nasal secretions collected from these children at two-week intervals were studied in detail. The mean levels of total proteins and immunoglobulins remained quite uniform throughout the test period with the exception of day 56 on which the levels were somewhat increased in the group receiving attenuated vaccine. (Table III). In all instances, the γA immunoglobulins were present in relatively greater concentration than the γG immunoglobulins.

Characterization of antibody in nasal secretions in both groups was accomplished by means of radioimmumodiffusion studies. Representative analyses are given in Table IV. In the only two specimens found to contain neutralizing antibody from two children immunized with inactivated vaccine, radioimmunodiffusion binding of virus was noted with the γG immunoglobulins in 1 case (CDA), and the γA and γG immunoglobulins in the other (DA). In secretions obtained from children immunized with attenuated vaccine found to contain neutralizing antibody activity, characterization of antibody by radioimmunodiffusion revealed it to be predominantly of the γA-variety (Table IV).

In a selected group of secretions, radioimmunodiffusion studies were performed using an antiserum specific for the secretory (11.4S) γA globulins. Analysis with this antiserum revealed positive binding in 5 secretions.

Characterization of Serum Antibody Response. Sera collected from the same children whose nasal secretions were analyzed were also studied by means of sucrose density gradient ultracentrifugation and radioimmunodiffusion studies (Table V). Analysis by these techniques revealed no significant differences in the distribution of serum antibody of children immunized with inactivated or with attenuated vaccines. Neutralizing antibody was detected in association with the γA, γG, and γM immunoglobulins.

The present findings suggest a significant difference in the humoral and local neutralizing antibody responses of children to immunization with inactivated and live measles virus vaccines. These responses are quite similar to these described with parainfluenza type 1 inactivated vaccine and live parainfluinza infection. (Annual report 1966-67). Immunization with inactivated vaccine appears to be as equally effective in stimulating serum antibody as the attenuated vaccine, however, antibody in nasal secretions appears regularly only after the use of attenuated measles virus vaccine.

Recent reports have appeared of unexpected reactions in recipients of inactivated measles virus vaccine who later received live vaccine or who are exposed to natural measles. Such children have been noted to develop atypical skin lesions and pneumonitis following measles or a localized Arthus reaction following live measles immunization. Such atypical responses have also been seen in children immunized with inactivated respiratory syncytial virus vaccine and who later acquire natural disease.

Two mechanisms have been suggested to explain these untoward reactions: one suggests delayed hypersensitivity, the other suggests Arthus type reaction.

The present findings would support the latter hypothesis. Recipients of inactivated measles vaccine appear to be unable to produce local IgA antibody in respiratory tract secretions although the serum antibody response is adequate.

Upon exposure to natural measles, or immunization with attenuated vaccine, the child would respond with viral replication in the respiratory tract or in the skin and the serum antibody response would be accelerated in an anamnestic response. When this happens, an unusual situation would be produced with both virus and antibody present at the same time. When this occurs immunologic injury would be produced by an antigen-antibody interaction at the site of viral replication, V_{12} lung or skin in a typical Arthus type reaction.

These observations have obvious relevance to the problem of immunoprophylaxis in viral respiratory tract disease. It is clear that vaccines which stimulate serum antibody selectively without respiratory tract antibody should not be used for at least 2 reasons. First, the presence of local IgA antibody seems to be of paramount value in protecting the host to viral infection as a first line of defense. Secondly, an imbalance may be created as a consequence of these vaccines which could lead to a state of altered reactivity following subsequent natural infection or the use of attenuated vaccine.

These studies will be continued over the coming year in animal models in order to gain further insight into the ability of attenuated vaccines or infection to stimulate respiratory antibody.

b) Characterization of serum and local antibody responses in recruits infected with adenovirus type 4.

A second major area of investigation of this laboratory over the past year has been the study and characterization of serum and nasal antibody following immunization and/or infection with adenovirus type 4.

The methods of gel filtration with Sephadex gel filtration (Figs. 3,4) have given partial information regarding the sequential appearance of antibody in serum and the characterization of nasal antibody (Annual reports 1965-66, 1966-67). The method of radioimmunodiffusion described in last year's annual report has been studied in greater detail with regard to sensitivity and specificity.

Tests of sensitivity. In order to assess the sensitivity of the radioimmunodiffusion method, sera obtained from volunteer study performed at Lorton Reformatory were studied. These sera were from men immunized orally with an adenovirus type 4 attenuated vaccine and challenged intra nasally at 21 days after immunization. Varying dilutions of serum and of radioactive virus antigen were set up in radioimmunodiffusion studies and end points determined as described previously (Annual report 1966-67). The unit of antigen or of antibody was arbitrarily defined as the highest dilution of either which gave a detectable line (2 +) on the radioautograph; this method is analogous to that employed in the standard Kolmer complement-fixation test used in many diagnostic virology laboratories. Representative analyses are given in Table VI. As can be seen, the method is extremely sensitive; dilutions of

antigen as high as 1:512 gave detectable activity. Similarly serum binding titers of 1:64 and 1:128 were found in sera with neutralizing titers of 1:10 and 1:320 respectively.

Tests of specificity These studies are now in progress but suggest that the antigen measured in this test is the group reactive (CF) antigen of adenovirus and not the serotypic specific antigen. These studies will be completed in the coming year's support and attempts will be made to further purify the reactive antigen by means of column chromatography or ultracentrifugation.

Salmonella studies

a) Human studies

Over the past year another major segment of this laboratory's effort has been directed toward the immunochemical study of humoral and local antibody responses in the human to infection and or immunization with various strains of Salmonella.

1) Characterization of serum and local antibody responses following natural infection.

The characterization studies of serum antibody in an infant and two adults following natural *S. typhosa* infection have been completed.

An additional four children who had been infected with Salmonella type B were identified. Sera and gastrointestinal specimens were obtained from these children and characterization studies performed as described in Annual Report 1966-1967.

These results are summarized in Tables VII-VIII.

Subjects The subjects were all children whose ages ranged from 1 to 7 years. All had Salmonella type B gastroenteritis and sera and gastrointestinal secretions were collected at varying intervals following recovery.

Methods. Radioimmunodiffusion binding was performed as described in last year's annual report with the exception that a purified *S. typhimurium* endotoxin was used which had been labeled with I^{131} rather than C^{14}.

In addition, blocking experiments were included in the tests as follows. Purified Fraction II, which consists mainly of γG immunoglobulins were added to the serum in a concentration of 500/ug/ml dilutions prior to the development of precipitin lines. In a similar fashion, purified E. *coli* and S. typhimurium endotoxin in a concentration of 2 ug/ml were each added to aliquots of serum prior to testing.

Results. The distribution of antibody in the unblocked serum specimens obtained at 2-3 weeks was generally found in association with the γM - and γG immunoglobulins occasionally with the γA immunoglobulins (C.P. and A.C.) Table VII. Following 3 weeks, most of the serum antibody was found in association with the γA and γG immunoglobulins. The blocking experiments showed that the addition of FrII could effectively remove all of the γG binding. There was no detectable

removal of antibody by the E. coli endotoxin. The removal of antibody by S. typhimurium endotoxin was not complete.

These results indicate that following natural infection with Salmonella a sequence of antibody distribution is seen in serum with earliest antibody of the γM, and γG and later predominantly with the γG and γA immunoglobulins.

Characterization studies of antibody in gastrointestinal secretions are given in Table VIII. Use of specific antisera directed toward each of the serum immunoglobulins and for the secretory (11 S) immunoglobulins and transport piece (T) revealed that Salmonella antibody can be detected in gastrointestinal secretions. In all cases it can be detected in association with γG-immunoglobulins in the duodenal fluids. The presence of antibody of the secretory 11S variety could be detected in the duodenal secretions of one child (A.C.). These results indicate that antibody in gastrointestinal secretions can be found following natural Salmonella infection and that in at least one instance it can be demonstrated to be of the 11 S secretory variety.

These results are in marked contrast to the results reported last year in which it was not possible to demonstrate the presence of local antibody in the nasal secretions of children immunized with killed Salmonella vaccines (Annual report 1966-67).(See below).

2. Characterization of serum antibody responses in children immunized with Salmonella vaccines.

Over the past year the studies of characterization of serum and local antibody responses in children immunized with Salmonella vaccines have continued. These studies have been performed in collaboration with Dr. William A. Altemeier, III and Col. Buescher at WRAIR. These studies have shown that relative inability of these inactivated vaccines to induce Secretory γA antibody. Secondly, most of the γM associated antibody in serum has been shown to be directed primarily toward the flagellar antigens. These studies will be continued over the coming year's support.

b) Animal studies.

Over the coming year's support, characterization of serum antibody responses to Salmonella and E. coli vaccines will be studied in the rabbit.

B. Other clinical studies related to the basic proposal supported by the contract

1. Herpes simplex encephalitis: brain biopsy and treatment with 5-iodo-2-deoxyuridine.

The clinical, virological, and pathological aspects in 4 children with herpes simplex encephalitis were studied: The diagnosis in each case was established by the direct isolation of virus from the brain. Three of the 4 children were diagnosed by viral isolation from postmortem brain tissue and in 2 of these, intranuclear inclusions and cytopathological changes indicated viral encephalites. In the child who survived, the diagnosis was established by brain biopsy, and a 6 day course of systemic 5-iodo-2-deoxyuridine was established. The role of the drug in the child's recovery is speculative but suggests that cautious trials of the drug in future cases may be indicated.

2. Postvaccinial lymphadenitis: a benign reaction of lymph nodes that can be misinterpreted as a malignant lymphoma.

These studies which were performed in collaboration with Dr. Robert J. Hartsock have compared the responses of lymph nodes to various vaccines and in lymphomas. These studies have revealed basic similarities in lymph node response after vaccination with the response in lymphoma and call attention to this fact in interpreting such responses.

Summary and Conclusions.

This is a definitive study of the biologic significance of antiviral antibody in order to assess its role in immunity in man. Control mechanisms and sequence data are being sought. The study of serum and local respiratory antibody responses has been performed in children immunized with inactivated and attenuated measles virus vaccines. The data suggest that although the distribution of serum antibody is quite similar following the use of both vaccines, respiratory γA- associated antibody is produced regularly only after immunization with attenuated vaccine. In addition, studies of characterization of nasal and serum antibody responses in recruits infected with adenovirus type 4 have continued as well as the study of responses of children infected with Salmonella. These studies have included the use of radioimmunodiffusion techniques.

IV Publications:

1. Bellanti, J.A., Artenstein, M.S. and Buescher, E.L.,: Radioimmunodiffusion studies of adenovirus antibody in human serum and nasal secretions. (in preparation).

2. Bellanti, J.A., Sanga, R.L., Klutinis, B., Brandt, B., and Artenstein, M.S. Antibody responses in serum and nasal secretions of children immunized with inactivated and attenuated measles virus vaccines (in preparation).

3. Bellanti, J.A. and Jackson, Anne L. Characterization of the Serum immunoglobulins to the somatic antigen of S. typhosa in an infant following intrauterine immunization. J. Pediat. 71: 783, 1967.

4. Altemeier, W.A., Bellanti, J.A., and Buescher, E.L.: Quantitation of γM antibody protein induced by routine typhoid immunization. To be presented at the Society For Pediatric Research, Atlantic City, N.J., May, 1968.

5. Walters, C.S., Bellanti, J.A., and Jackson, A.L., Cells releasing antibody to E. coli - antigens Fed. Proc. 27:735, 1968.

6. Bellanti, J.A., Role of Local gamma-A-immunoglobulins in immunity. Am. J. Dis. Child. 115:239, 1968.

7. Bellanti, J.A., Guin, G.H., Grassi, R.M. and Olson, L.C. Herpes simplex encephalitis, brain biopsy, and treatment with 5-iodo-2-deoxyuridine. J. Pediat. 72:266, 1968.

8. Hartsock, R.J. and Bellanti, J.A., Postvaccinial lymphadenitis: a benign reaction of lymph nodes that can be misinterpreted as a malignant lymphoma. GP (in press) 1968.

TABLE I

INDIVIDUAL GROUPS OF CHILDREN RECEIVING INACTIVATED (K) OR LIVE, ATTENUATED (L) MEASLES VIRUS VACCINES

Group	No. of Children	Pre-immunization Geometric Mean Serum Titers	Type of Vaccine
Seronegative			
I	7	<1	K
II	9	<1	L
Seropositive			
III	5	89	K
IV	3	155	L
Total	24		

TABLE II

INFLUENCE OF TYPE OF MEASLES VACCINE ON PRODUCTION OF ANTIBODY IN SERUM AND IN NASAL SECRETIONS

Group	Specimen	Frequency of antibody detection by day						
		0	14	28	42	56	70	84
Inactivated	serum	0/7*	0/6	1/7	6/7	6/7	3/4	2/2
	nasal	0/7	0/6	0/7	0/6	1/6	1/4	0/2
Live, attenuated	serum	0/9	8/8	9/9	9/9	9/9	9/9	5/5
	nasal	0/9	3/8	2/6	3/8	5/8	2/9	0/5

*fraction refers to number of specimens showing presence of neutralizing antibody over the total number of specimens tested

TABLE III

MEAN TOTAL PROTEIN AND IMMUNOGLOBULIN CONCENTRATIONS IN NASAL SECRETIONS OF CHILDREN IMMUNIZED WITH MEASLES VACCINES

Group	No. of Children	Days After Immunization	Total protein	γA	γG
SERONEGATIVE			mgm/100 ml	mgm/100 ml	mgm/100 ml
I	7	0	186	20	<10
		14	378	34	26
		28	280	22	12
		42	322	23	<10
		52	293	26	<10
		70	356	23	<10
		84	70	22	<10
II	9	0	186	25	<10
Live, attenuated		14	126	20	<10
		28	618	40	13
		42	304	31	6
		56	1309	80	25
		70	349	27	<10
		84	391	26	<10
SEROPOSITIVE					
III	5	0	678	47	17
		14	487	38	<10
(Inactivated)		28	285	26	12
		42	693	37	17
		56	598	44	16
		70	315	29	11
		84	225	29	12
IV	3	0	52	10	<10
(Live, attenuated)		14	229	22	10
		28	521	28	12
		42	384	32	19
		70	772	37	15
		84	310	23	<10

TABLE IV

CHARACTERIZATION OF MEASLES ANTIBODY IN NASAL SECRETIONS BY RADIOIMMUNODIFFUSION BINDING STUDIES

Subject	Interval After Immunization	Neutralizing titer	Total Protein	γA	γG	Radioimmunodiffusion γA Binding	γG
Inactivated vaccines			mgm/100ml	mgm/100ml	mgm/100ml		
D.A.	42	<1	384	27	9	±	0
	56	<1	736	41	13	±	0
	70	8	400	24	10	±	±
C.D.A.	28	<1	38	18	0	0	0
	42	<1	N.T.	N.T.	N.T.	N.T.	N.T.
	56	2	500	N.T.	N.T.	0	±
Live, Attenuated vaccine							
L.L.	14	4	312	20	2	+	0
	42	2	352	30	3	+	0
	70	2	572	27	6	+	0
R.M.	14	<1	64	N.T.	6	0	0
	28	<1	448	34	12	0	0
	42	<1	N.T.	25	8	0	0
	70	<1	408	27	12	0	0

TABLE V

CHARACTERIZATION OF MEASLES ANTIBODY IN SERUM BY ULTRACENTRIVUGATION AND RADIOIMMUNODIFFUSION

SUBJECT	Interval after Immunization (Days)	Neutralizing Antibody Titer	UCF		Radioimmunodiffusion Binding			
			19S	7S	γG	γA	γM	
Inactivated Vaccinees								
D.A.	42	8	1+	4+	32	2	±	
	56	32	1+	4+	16	1	+	
	70	1024	2+	4+	≥32	2	2	
C.D.A	28	16	+	+2	≥32	1	±	
	42	256	+	+4	≥32	0	2	
	56	64	+	+4	≥32	0	±	
Live Vaccinees								
L.L.	14	64	+	0	32	0	1	
	28	64	+2	+	32	0	2	
	42	128	±	+4	≥32	0	1	
	70	128	0	+4	≥32	0	0	
R. M.	14	32	2	+2	16	2	4	
	28	64	±	+2	≥32	0	1	
	42	64	0	+3	≥32	2	2	
	70	128	+	+4	≥32	0	1	

TABLE VI

TESTS OF SENSITIVITY OF THE RADIOIMMUNODIFFUSION METHOD

Serum	Neut titer	Day after immunization	Grid titration							
			Serum dilution							
			Antigen dil	2	4	8	16	32	64	128	256
Br.	10	21	8	2+	2+	2+	2+	2+	2+	0	0
			32	2+	2+	2+	2+	2+	1+	0	0
			128	1+	1+	1+	1+	1+	1+	0	0
			256	1+	1+	1+	1+	1+	1+	0	0
			512	±	±	±	±	±	±	0	0
Ga.	320	48	8	+2	+2	+2	+2	+2	+2	0	
			32	+2	+2	+2	+2	+2	+2	+1	
			128	+2	+2	+2	+2	+2	+2	+2	
			256	+2	+2	+2	+2	+2	+2	+2	
			512	+1	+1	+1	+1	+1			

TABLE VII

CHARACTERIZATION OF SERUM ANTIBODY RESPONSE BY RADIOIMMUNODIFFUSION BINDING FOLLOWING NATURAL SALMONELLA TYPE B INFECTIONS

Subject	Age	Interval (Days)	RADIOIMMUNODIFFUSION						BINDING			TITERS		
			Unblocked			FR II			E.colis			S. typhimurium		
			G	A	M	G	A	M	G	A	M	G	A	M
C. P.	3 yr.	13	256	16	8	<2	32	8	256	32	4	512	16	8
		25	128	8	4	<2	8	8	128	16	4	128	8	4
		52	256	16	<2	<2	16	8	256	16	<2	256	16	<2
N. G.	15 mos.	~ 360	256	16	<2	<2	16	<2	N.T.	N.T.	N.T.	64	8	<2
A. C.	7 yrs.	14	32	<2	16	<2	<2	16	N.T.	N.T.	N.T.	32	<2	<2
		90	32	32	<2	<2	32	<2	N.T.	N.T.	N.T.	32	16	<2
M.M.	1 yr.	~ 300	512	16	8	<2	16	8	N.T.	N.T.	N.T.	128	8	4

TABLE VIII

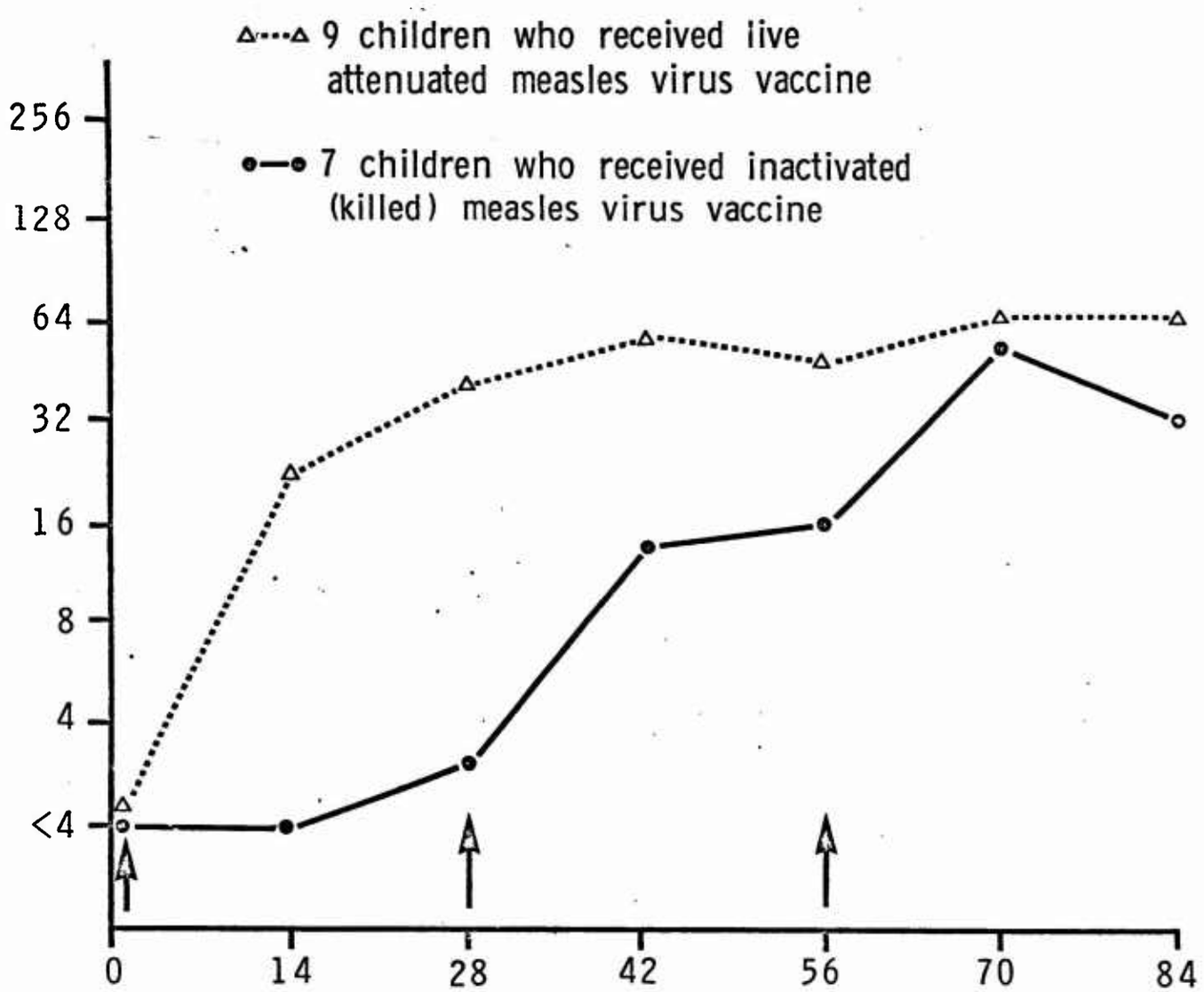
CHARACTERIZATION OF GASTROINTESTINAL ANTIBODY BY IMMUNOCHEMICAL AND RADIOIMMUNODIFFUSION BINDING TECHNIQUES FOLLOWING NATURAL SALMONELLA TYPE B INFECTIONS

Subject	Specimen*/	Interval (Days)	QUANTITATION (mgm/100ml)						RADIOIMMUNODIFFUSION BINDING				
			T.P.	γG	γA	γM	11S	T	γA	γG	γM	11S	T
C.P.	G	13	172	<10	<10	<10	0	0	0	0	0	0	0
	D	13	188	14	<10	<10	0	0	0	+	0	0	0
	G	25	608	38	40	<10	0	0	0	0	0	0	0
	D	25	128	20	<10	<10	0	0	0	+	0	0	0
N.G.	G	360	576	<10	24	<10	0	0	0	0	0	0	0
	D	360	832	<10	24	<10	0	+	0	0	0	0	+
A.C.	G	90	512	<10	24	<10	0	0	0	0	0	0	0
	D	90	2176	<10	24	60	+	+	+	+	+	+	+

*G = gastric

D = duodenal

MEASLES NEUTRALIZING ANTIBODY RESPONSE IN SERUM FOLLOWING IMMUNIZATION



WITH MEASLES VACCINES

FIG. I

MEASLES NEUTRALIZING ANTIBODY RESPONSE IN NASAL SECRETIONS FOLLOWING IMMUNIZATION

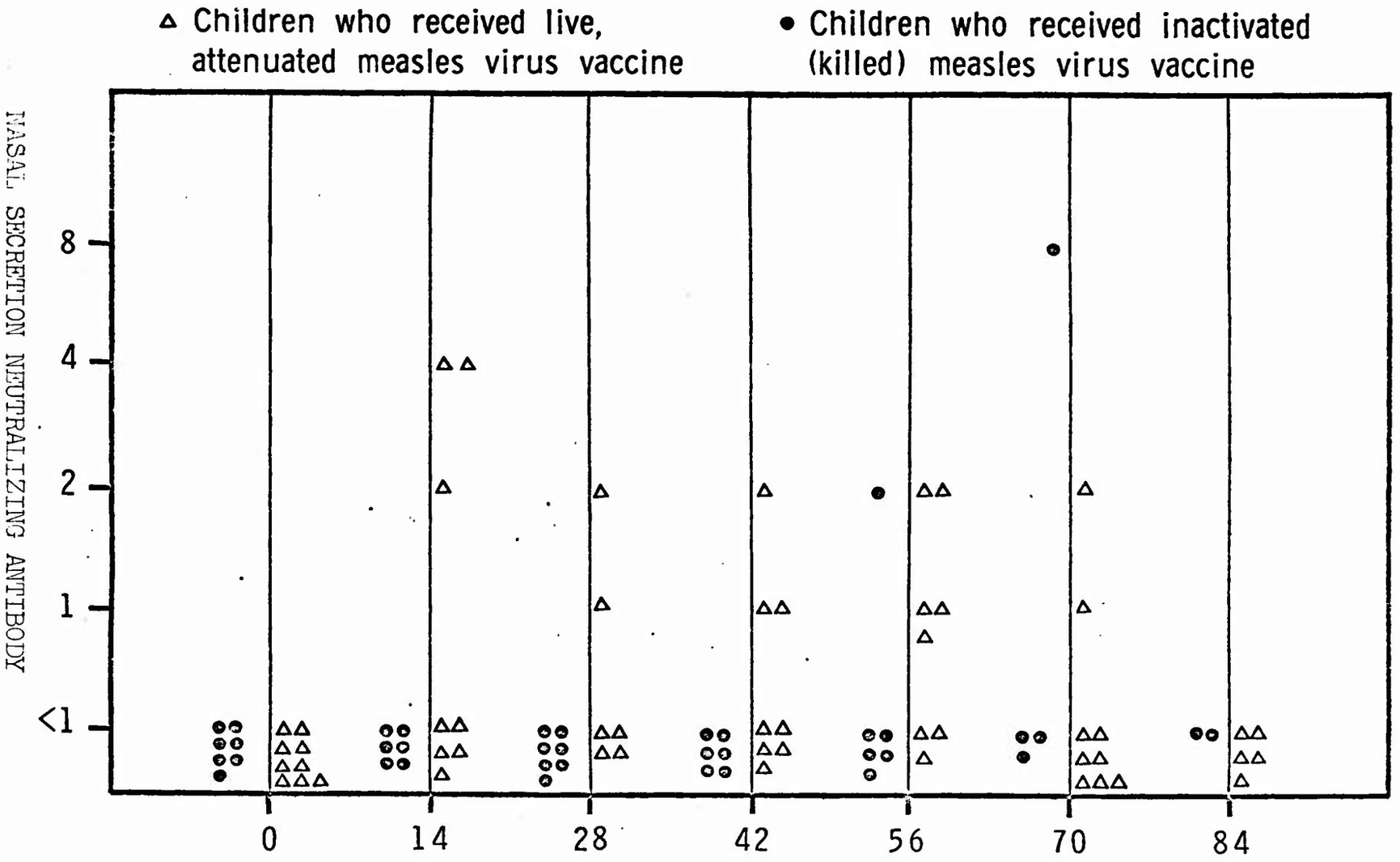


FIG. II

FIG. III

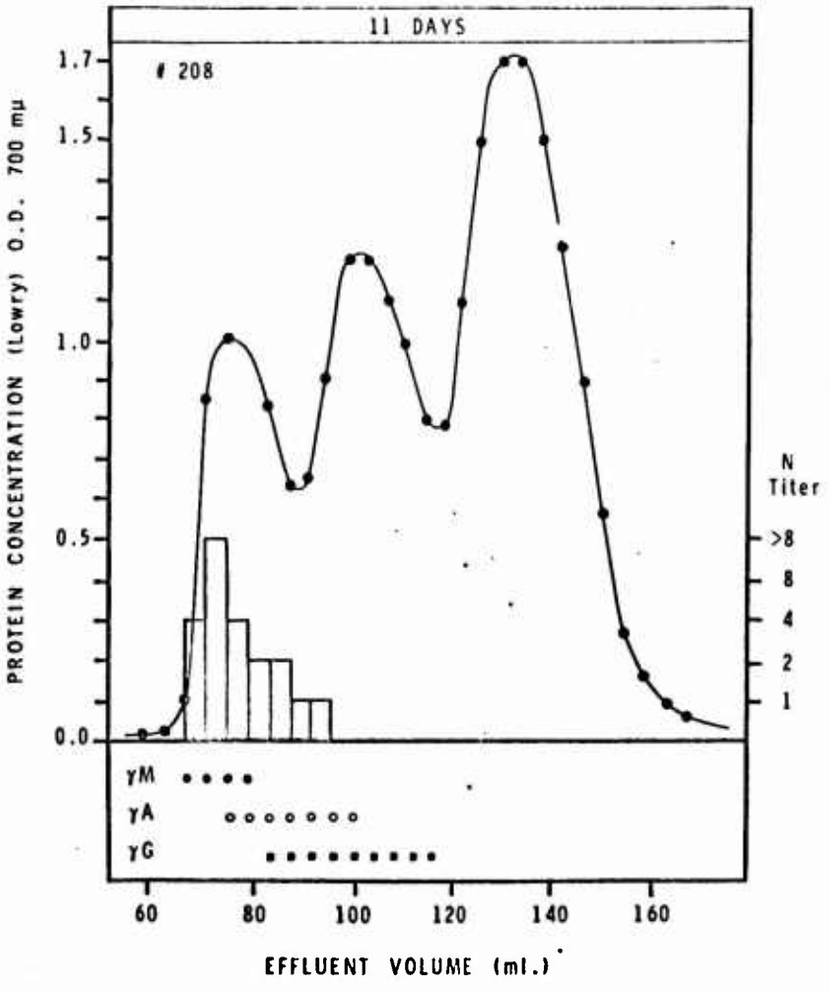
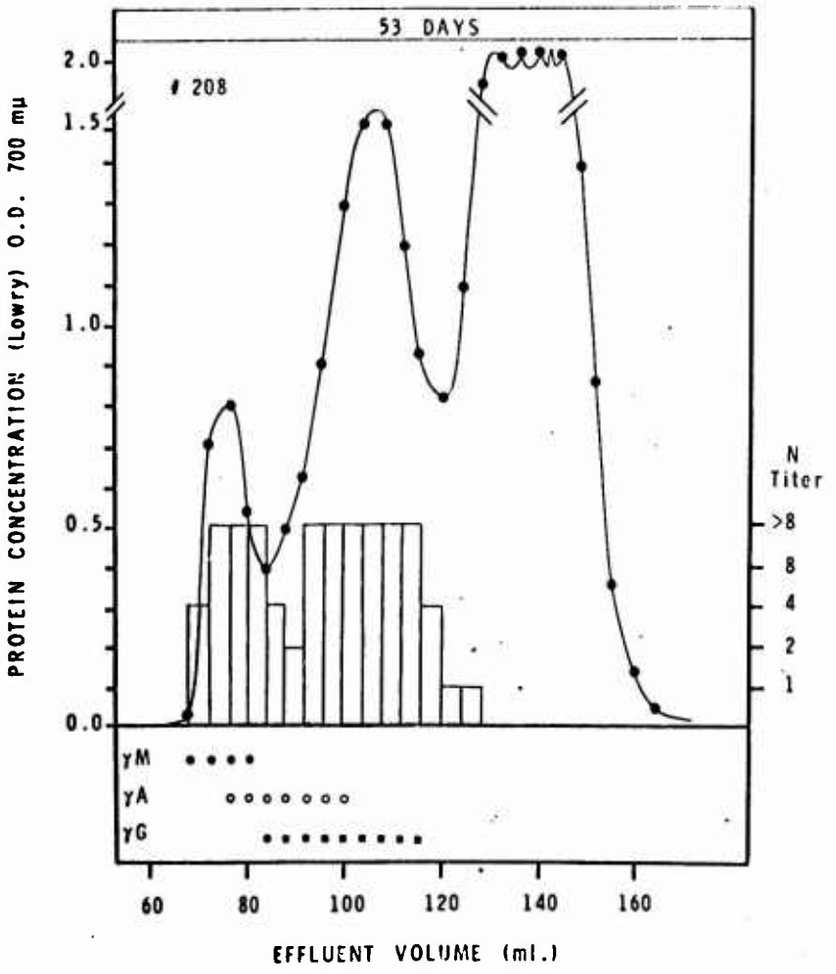

11 DAYS
208
PROTEIN CONCENTRATION (Lowry) O.D. 700 mμ
1.7
1.5
1.0
0.5
0.0
N Titer
>8
8
4
2
1
γM
γA
γG
60
80
100
120
140
160
EFFLUENT VOLUME (ml.)

FIG. IV


53 DAYS
208
PROTEIN CONCENTRATION (Lowry) O.D. 700 mμ
2.0
1.5
1.0
0.5
0.0
N Titer
>8
8
4
2
1
γM
γA
γG
60
80
100
120
140
160
EFFLUENT VOLUME (ml.)

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 044490	CSCRD-103

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

10a. CURRENT NUMBER/CODE
61130011 3A013001A91C 00 106

10b. PRIOR NUMBER/CODE

11. TITLE (U) CYTOGENETIC AND METABOLIC DETERMINANTS IN THE EVOLUTION OF CELL POPULATION FOLLOWING INJURY

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	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE c. DATE d. AMOUNT NA	PRIOR FY 68	0	10
		CURRENT FY 69	0	10

19. GOVT. LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION
CLINICAL MEDICINE

22. COORDINATION
NA

23. KEYWORDS
CELLULAR GENETICS, CELL CULTURE, CHROMOSOMES, FREEZING, INJURY, STRESS, PHYSIOLOGY.

24.

(U) TECH OBJECTIVE - TO INVESTIGATE GENETIC AND METABOLIC ALTERATIONS IN CELL POPULATIONS SURVIVING INJURY. AMONG SUCH ALTERATIONS, THOSE RESPONSIBLE FOR THE PROGRESSIVE DETERIORATION OBSERVED IN INDIVIDUALS EXPOSED TO IONIZING RADIATION AND FOR THE DIFFICULTIES ENCOUNTERED IN THE USE OF FROZEN CELLS AND TISSUES FOR GRAFTING HAVE MILITARY SIGNIFICANCE AND, ACCORDINGLY, CONSTITUTE THE SPECIFIC OBJECTIVES OF THIS PROJECT.

25. (U) APPROACH- TO MAXIMIZE EXPERIMENTAL CONTROL, IN VITRO CULTURES OF PARTIALLY SYNCHRONIZED FIBROBLASTIC CELLS WERE FROZEN IN THE PERIOD BEFORE (G1) AND DURING (S) THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID (DNA) AND THE INJURY THUS INDUCED WAS ANALYZED IN TERMS OF POST-THAW VIABILITY, CHROMOSOMAL COMPLEMENT AND DIVISION CYCLE KINETICS.

26. (U) PROGRESS - JUL 67 THRU JUN 68 CELL POPULATIONS FROZEN DURING G1, EXHIBITED UPON THAWING A DECREASE OF THEIR VIABILITY BY 50-60 PERCENT, AN INCREASE OF THE RELATIVE NUMBER OF LOW PLOIDY CELLS, A DISPERSION OF RELATIVE FREQUENCIES WITHIN THE MODAL RANGE OF THEIR CHROMOSOME COMPLEMENT RESULTING IN A DECREASE OF THE 48 CHROMOSOME STEMLINE FROM 32 PERCENT OF THE POPULATION TO 16 PERCENT, AND AN ELONGATION OF THE LARGEST TELOCENTRIC CHROMOSOME. CELL POPULATIONS FROZEN DURING S EXHIBITED A DECREASE OF THEIR VIABILITY BY 10-20 PERCENT, A SMALLER INCREASE OF THE RELATIVE NUMBER OF LOW PLOIDY CELLS AND MAINTENANCE OF THE 48 CHROMOSOME STEMLINE AT 32 PERCENT, NORMAL LENGTH OF THE LARGEST TELOCENTRIC CHROMOSOME AND AN INCREASE OF THE RELATIVE NUMBER OF LARGE METACENTRIC AND SUBMETACENTRIC CHROMOSOMES AT THE EXPENSE OF THE SMALLER CHROMOSOMES OF THE SAME TYPE. THESE RESULTS INDICATE THAT THE SELECTIVE PRESSURE EXERCISED BY FREEZING INJURY WOULD TEND TO FAVOR CELLS IN THE S PERIOD. 27. THIS CONCEPT IS ALSO SUPPORTED BY PRELIMINARY FINDINGS SUGGESTING A NEARLY NORMAL DIVISION CYCLE FOR CELLS FROZEN DURING S PERIOD, WHILE CELLS FROZEN DURING THE G1 PERIOD APPEAR TO BE GREATLY DELAYED. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		6R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In thousands)	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 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: Robert J. Werrlein, M.S. and J. R. C. Brown, Ph.D.

Description.

While the mechanisms of cell growth and regeneration responsible for the repair of tissues following injury have been under intensive investigation, not enough attention has been given to the genetic and metabolic alterations of cell populations surviving injury. Yet, such alterations are of great military interest as evidenced by the fact that they are responsible for the progressive deterioration observed in individuals exposed to ionizing radiation and for the difficulties encountered in the use of frozen cells and tissues for grafting (cf. the work of Van Bekkum et al in Holland supported by Army Contract DAJA37-68-C-0081 on the use of frozen bone marrow preparations in the therapy of radiation injury).

In order to maximize experimental control, accuracy of quantitation and analytical resolution, a well defined in vitro culture system is used in this department to study fibroblastic cell populations following freezing and radiation injury.

Progress.

During the current year these studies were focused on freezing partially synchronized cell populations before (G1 period) and during (S period) the synthesis of deoxyribonucleic acid (DNA) and analyzing the resulting injury in terms of cell viability, modification of the chromosomal complement of the cells, and alterations of the time parameters of their division cycle. The respective methods used were cell counts and plating efficiency, chromosome counts and caryotyping, and tritiated thymidine autoradiography.

It was found that cell populations frozen during the period preceding DNA synthesis (G1 period) exhibited upon thawing a decrease of their viability by 50-60%, an increase of the relative number of low ploidy cells, a dispersion of relative frequencies within the modal range of their chromosome complement resulting in a decrease of the 48 chromosome stemline from 32% of the population to 16%, and an elongation of the largest telocentric chromosome which serves as an internal standard for the caryotyping of these cells. Cell populations frozen

during DNA synthesis (S period) exhibited upon thawing a decrease of their viability by 10-20%, a smaller increase of the relative number of low ploidy cells and a lesser dispersion within the modal range resulting in maintenance of the 48 chromosome stemline at 32%, maintenance of the length of the largest telocentric chromosome within control values, and an increase of the relative number of large metacentric and submetacentric chromosomes at the expense of the smaller chromosomes of the same type.

Among the viable cells of populations frozen during the G_1 period as well as of those frozen in S there appear to be some which give rise to clones of fibroblasts which are grossly abnormal in appearance, while others produce clones which maintain the typical fibroblast morphology. It is not yet known whether such cells can survive indefinitely and complete successfully, thereby altering the original population. On the other hand it would appear that the results obtained up to date indicate that the selective pressure exercised by freezing injury would tend to favor cells in the S period. This concept is also supported by preliminary findings relative to the time parameters of the division cycle of cells originating in cultures frozen during the G_1 and the S periods. Cells frozen while in G_1, generally have shown substantial increase in generation time after thawing. Where significant increases in generation time did occur, the greatest effect has been observed in the extension of the DNA synthetic phase (S), followed closely by the extension of the pre-DNA synthetic phase (G_1) with very little change being introduced by increases in either mitotic duration* of the period which follows the synthesis of DNA (G_2). In contrast, cells frozen while in the DNA synthetic phase (S) show generation times which are nearly normal thus gaining an advantage over G_1 frozen cells, which is in addition to their viability and chromosomal complement characteristics previously described. Further work aiming to test the validity of this concept is in progress.

Summary and Conclusions.

The objective of this work unit is to investigate genetic and metabolic alterations in cell populations surviving injury. Among such alterations, those responsible for the progressive deterioration observed in individuals exposed to ionizing radiation,and for the difficulties encountered in the use of frozen cells and tissues for grafting, have military significance and, accordingly, constitute the specific objectives of this project. To maximize experimental control, in vitro cultures of partially synchronized fibroblastic cells were frozen in the period before (G_1) and during (S) the synthesis of deoxyribonucleic acid (DNA) and the injury thus induced was analyzed in terms of post-thaw viability, chromosomal complement and division cycle kinetics.

Cell populations frozen during G_1, exhibited upon thawing a decrease of their viability by 50-60%, an increase of the relative

* or the duration

number of low ploidy cells, a dispersion of relative frequencies within the modal range of their chromosome complement resulting in a decrease of the 48 chromosome stemline from 32% of the population to 16%, and an elongation of the largest telocentric chromosome. Cell populations frozen during S exhibited a decrease of their viability by 10-20%, a smaller increase of the relative number of low ploidy cells and maintenance of the 48 chromosome stemline at 32%, normal length of the largest telocentric chromosome and an increase of the relative number of large metacentric and submetacentric chromosomes at the expense of the smaller chromosomes of the same type. These results indicate that the selective pressure exercised by freezing injury would tend to favor cells in the S period. This concept is also supported by preliminary findings suggesting a nearly normal division cycle for cells frozen during S period, while cells frozen during the G_1 period appear to be greatly delayed.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA6491	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 68	B. COMPLETED 01 07 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 109

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) CHROMOSOME FUNCTION AND INJURY

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. PROCUREMENT METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
A. GRANT	11 64 a. NUMBER DA 49 193 MD 65 G148 c. TYPE Y.GRANT d. AMOUNT $40,200	PRIOR FY 67	1	13
		CURRENT FY 68	0	3

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INVESTIGATORS PRINCIPAL: BROWN, J. R. C.
ASSOCIATE: GLINDS, A. C.
TEL: 301-927-3800-EXT 210 TYPE: DA

21. TECHNOLOGY UTILIZATION: CLINICAL MEDICINE

22. COORDINATION: NA

23. KEYWORDS: CELL CULTURE, CYTOGENETICS, THERMAL STRESS, INJURY, DNA, RNA, PROTEIN, DIFFERENTIATION.

24. (U) TECH OBJECTIVE - TO COMPLEMENT THE IN-HOUSE WORK UNIT ON CYTOGENETIC AND METABOLIC DETERMINANTS IN THE EVOLUTION OF CELL POPULATIONS, THROUGH THE STUDY OF THE EFFECTS OF ADVERSE CONDITIONS, SUCH AS ELEVATED TEMPERATURE IN THE FEVER RANGE, HIGH DENSITY CELL POPULATIONS, OR LIMITED NUTRITIONAL SUPPLY, ON THE REPLICATION OF CELLS IN CULTURE. SUCH INFORMATION IS OF SIGNIFICANCE IN WOUND HEALING OR IN MAINTENANCE OF CELLULAR RESPONSE TO INFECTION.

25. (U) APPROACH- SUSPENSION CULTURES OF CELL LINES OBTAINED FROM CLONED L-929 MOUSE FIBROBLASTS WERE EXPOSED TO ELEVATED TEMPERATURE (40.5 DEGREES C.) FOR 12 HOURS. DURING AND FOLLOWING TREATMENT, DETERMINATIONS WERE MADE OF CELL VIABILITY, REPLICATION RATES, RATES OF SYNTHESIS OF DNA AND PROTEIN, AND LEVELS OF MITOTIC ACTIVITY. KARYOTYPE ANALYSIS OF THE CELL LINE BEFORE AND AFTER TREATMENT WAS ALSO MADE. SIMILAR CULTURES ARE ALLOWED TO GROW WITH DAILY MEDIA RENEWAL UNTIL A STATIONARY POPULATION LEVEL OF HIGH DENSITY (UP TO 12 MILLION CELLS/ML.). CELL COUNTS, MITOTIC INDEX AND RATE OF RNA SYNTHESIS/CELL ARE DETERMINED IN THE LOG AND STATIONARY PHASES.

(U) PROGRESS - JUL 67 THRU MAY 68 IN CULTURES SUBJECTED TO HEAT TREATMENT IT WAS FOUND THAT DURING EXPOSURE TO HIGH TEMPERATURE THERE WAS AN EXTREME REDUCTION IN THE INCORPORATION OF TRITIUM LABELED THYMIDINE AND LEUCINE, INDICATING REDUCED LEVELS OF SYNTHESIS OF DNA AND PROTEIN. CONSIDERABLE CELL DEATH WAS ALSO OBSERVED DURING HEAT TREATMENT. CULTURES RETURNED TO NORMAL TEMPERATURES DEMONSTRATE A LAG PHASE OF ABOUT FOUR HOURS, FOLLOWED BY A BURST OF DNA SYNTHESIS, SUGGESTING THAT THE CELLS MAY HAVE REVERTED TO G-1 PRIME PHASE. A WAVE OF MITOSES 18 HOURS AFTER HEAT TREATMENT INDICATES CONSIDERABLE SYNCHRONIZATION OF THE CELL CYCLES HAD BEEN INDUCED. ANALYSES OF KARYOTYPES OF SURVIVING CELLS DID NOT DEMONSTRATE ANY MARKED CHANGE IN CHROMOSOMAL COMPLEMENT. ANALYSIS OF RESULTS OF PROLONGED HIGH DENSITY CULTURE ON RNA SYNTHESIS ARE CURRENTLY BEING CONDUCTED, BUT NO CONCLUSIONS HAVE BEEN REACHED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	29.	28. 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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 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, Ph.D.
Associate: Lloyd T. McAtee, M. A., James M. Vail, M.S., Robert Simpkin, B.S., and Andre Glinos, M.D.

Description:

This project was designed as a part of, and a supplement to, the Walter Reed Army Institute of Research In-House Independent Project entitled "Cytogenetic and Metabolic Determinant in the Evolution of Cell Populations Following Injury." The research reported here was directed toward an investigation of the temporary and prolonged alterations in growth pattern, metabolic activity, and genetic composition, or expression, of cells subjected to controlled sub-lethal injury. Primary attention during the past year has been devoted to investigation of the effects of thermal stress on cell replication, synthesis of DNA and protein, and alteration of karyotype. Secondary attention has been devoted to investigation of the effects of high population density, or media depletion, on the level of high energy nucleotide pools in the cells (as reported in the Annual Report for 1966-'67).

In all investigations in our laboratory the cells under investigation were derived from a cloned line of NCTCL-929 mouse fibroblast. This clone (WRL-10-A) was isolated by the Department of Cell Physiology, WRAIR. A sub-line of this clone (SU-4) was obtained by our laboratory for use in these experiments. This

sub-line has chromosome modal range of 50-60 with about 90% of the cells in this range. Suspension cultures of these cells were grown in minimum essential medium plus 10% horse serum, 1% l-glutamine and with antibiotics added. Cultures were incubated at 35.5° C in 5% CO_2 in air with controlled humidity. Logarithmic growth was maintained by renewal of the culture media each 24 hours and cutting the population back to $4x10^5$ cells/ml. Under these conditions the majority of cells demonstrate a generation time of 18 to 20 hours.

Progress:

Progress in research during the past year will be discussed under the various projects under investigation.

I. Reaction of cultures to thermal stress in the fever range. For heat treatments, 180 ml sub-cultures were transferred from the stock culture into double walled spinner flasks, with temperature controlled water circulated through the outer jacket. One control at 35.5° C and two experimentals at 40.5° C were maintained by circulating water from two constant temperature water baths. The spinner flasks were placed on a single multiple stirrer to insure equal agitation. 5% CO_2 in air was supplied to the cultures throughout treatment. A thermo-probe inserted within a sterile pyrex sleeve was submerged into each of the cultures, and temperatures were monitored and recorded using a scanning YSI model 47 telethermometer and a YSI model 80 recorder. This permitted a continuous record of pre-treatment, 12 hour heat shock, and recovery. Samples were removed at selected intervals for determination of cell count, mitotic index, and ability to incorporate tritiated thymidine, or tritiated leucine.

A. Effect of thermal stress on cell replication and DNA synthesis.

Analysis of results from experiments performed in the above manner gave the following results:

1. As indicated by Figure 1, the growth rate of the control cultures in each case showed normal increases throughout the experiment, while the experimental cultures exposed to 40.5° C demonstrated negative growth rates with the population declining from an initial concentration of approximately 4×10^5 cells/ml to a final concentration of approximately 1.7×10^5 cells/ml at the end of the 12 hour heat stress. During the first nine hours (hours 12-21) following return of the heat treated cells to 35.5° C the culture showed a continued slight drop in cell count, indicating the presence of some damaged cells. The relative growth rates of control and experimental cultures in eight repeat experiments is analyzed in Figure 2. This bar graph shows the growth rates during and following heat treatment. During the first six hours of heat shock experiments, control cultures showed an average growth rate of 1.16, while experimental cultures exhibited a negative (-1) growth rate of 0.62. From 6 through 12 hours experimental culture demonstrated a growth rate of 0.73, while controls averaged a growth rate of 1.20. From the termination of heat shock at 12 hours through hour 21, experimental cultures exhibited a growth rate of 0.95 while controls showed a growth rate of 1.40.

Beginning at hour 24 through hour 45, control and experimental cultures showed nearly equal growth rates with control cultures averaging 1.90 and experimental cultures averaging a slightly higher growth rate of 2.00. Analyses of variance showed a significant difference ($p<.01$) among control and experimental cultures at each time interval studied from hour 0 through 21, while no significant difference was shown between control and experimental cultures from hour 24 through 45.

Results of determinations of mitotic rates are shown in Figure 3. Within one hour after initiation of heat shock, cell division in the experimental cultures was inhibited and remained inhibited until hour 21, while control cultures maintained an average division rate or mitotic index of 1 plus. From hour 21 through 24 the experimental cultures showed a slight increase in mitotic index. Beginning at hour 24 experimental cultures showed a rapid increase in the mitotic index which culminated at hour 30 with a mitotic index of 3.81, while control cultures maintained an average mitotic index of 1. By hour 33 the mitotic index of experimental cultures had decreased to approximately 1.2, similar to the mitotic index of control cultures. Analyses of variance performed on data from initiation of heat treatment through hour 33 showed a highly significant difference ($p<.001$) between control and experimental cultures.

DNA synthesis was analyzed at time intervals during and following heat shock by removing 10 ml samples from control and experimental cultures, equilibrating at 35.5° C and pulse labeling with tritiated thymidine for ten minutes. The cells were fixed and stained; then autoradiographs were prepared. Cells were considered labeled if they contained three or more grains per nucleus. The results of these labeling experiments are shown in Figure 4. During the entire experiment one nuclei from control cultures averaged 45% labeling. At initiation of heat shock 43% of the experimental nuclei incorporated the label. During heat shock the number of nuclei incorporating the label was reduced to 35% at 1 hour, 10% at 2 hours, 7% at 6 hours and 6.5% by 12 hours. For four hours after the termination of heat shock less than 10% of the experimental nuclei were labeled. Beginning at hour 16, or 4 hours after termination of heat shock, experimental nuclei showed a sharp increase in percent labeled nuclei to 47% labeled by hour 24 versus 44% for control nuclei. After hour 24 experimental cultures exhibited a decrease in percent labeled nuclei to 23% by hour 30.

A comparison of the results from determinations of mitotic index and thymidine H^3 incorportion show that during the exposure of the cells to 40.5° C there is an inhibition of both DNA replication and cell division. Following the return of the heat treated cells to 35.5° C

there is a lag period of 3 to 4 hours before resumption of DNA synthesis. Cell division remains at a very low level until approximately 18 hours when there appears a wave of mitosis. It appears, therefore, that those cells surviving the heat treatment have either been halted in the pre-DNA-synthesis stage (G_1) or have reverted to this stage as a result of degredative changes to enzyme systems or nucleic acids, and must pass through DNA synthesis (S phase) prior to division. Furthermore, the appreciable lag period prior to beginning of DNA syntheses indicates that some of the enzyme "machinery" for synthesis may require rebuilding following the rigorous heat stress. These results are in keeping with those reported by Byfield and Sch[illegible] (Science 153:1504, 1967), Rao and Engelberg (Chap. 17 in Cell Synchrony, Cameron and Padilla, Eds., Academic Press, 1966) and Ossovski and Sachs (Proc. Nat. Acad. Sci. 58:1940, 1967), in the indication of the effects of thermal shock on inhibition of nucleic acid synthesis and production of partial synchrony in cell populations.

In our experiments it has been demonstrated that heat treated cells following recovery do not demonstrate noticeable differences in growth pattern from the control cultures. Preliminary analyses of the karyotypes of cells surviving the period of thermal stress do not demonstrate obvious alterations from the controls.

B. Effect of Thermal stress on protein synthesis.

Using the same experimental set up as that described above, experiments have been in progress to determine the relative rates of protein synthesis in cells during and following thermal stress, compared to control cultures. Starting at the beginning of heat treatment (0 hours) samples were removed at 3 hour intervals for cell count and for pulse labeling with tritiated leucine. For the pulse label, 10 ml suspension cultures were equilibrated at 35.5° C, then 3μ C of ^{3}H-leucine was added. At the end of a 30 minute incubation period the cells were quickly cooled to 0° C in an ice bath, washed twice in Hank's Salt Solution, then extracted with 10% trichloroacetic-acid. The protein precipitate was dissolved in 0.5N KOH and counted in a Packard scintillation counter.

Results to date from these experiments have been somewhat erratic, however, on a counts/minute/cell basis it appears that ^{3}H-leucine incorporation is reduced during exposure to 40.5° C to approximately one third of the value of the control cultures. Cultures exposed to the heat treatment of 12 hours and then returned to incubation at 35.5° C show a rate of ^{3}H-leucine incorporation at 15 hours (3 hours post heat treatment) approximating that of the controls Thus it appears that during the DNA synthetic lag period there is a recovery of the rate of protein synthesis.

II. High Energy nucleotide pools of cells in suspension cultures during logarithmic, semi-starvation and stationary phases of growth.

During the early part of this year, work on this

project continued as described in the annual report for 1966-'67. The investigation was concluded with the presentation of a doctoral thesis by James M. Vail entitled: Relation of Energy Metabolism to Growth and Differentiation of L-Cell Suspension Cultures. A copy of this dissertation is attached to this report.

Conclusions:

A. Experiments on exposure of logarithmically growing L-cells in suspension culture to elevated temperatures (40.5° C) demonstrate that:

1. At this temperature there is an inhibition of synthesis of both DNA and protein.

2. Concurrent with, and probably resulting from, this synthetic inhibition there is a delay in cell division during the period of heat treatment and for approximately one cell generation (18 hours) following heat treatment. At the end of the 18 hour lag of cell division a wave of mitosis occurs indicating that a partial synchrony has been established.

3. In the recovery period following heat treatment, protein synthesis has apparently returned to normal by three hours post-treatment while synthesis of DNA demonstrates a somewhat longer lag period.

4. Observations listed in 2 and 3 above suggest that during exposure to elevated temperature, essential enzyme systems may be destroyed and must be resynthesized prior to resumption of the normal cell growth cycle.

D. Experiments on high density cultures, or cultures in partially depleted media, demonstrate that under these conditions there is a decrease in the level of intracellular ATP to less than half the value found in logarithmically growing cells. Concurrent with this reduced level of ATP there is a marked decrease of cellular replication and a marked increase in the nucleotide complex, uridine-5'-N-acetyl glucosamine-galactosamine. This compound may serve as a precursor in the synthesis of mucopolysaccharides. The decrease in ATP level is not offset by increases in ADP nor AMP. It is suggested that under sub-optimal growth conditions there may be a shift of active metabolic pathways from cellular replication to activities comparable to differentiation in somatic cells.

Publications:

1. Vail, J. M., J. R. C. Brown and A. D. Glinos, Acid soluble nucleotides in L-cell suspension cultures in various phases of growth. J. Cell Biol 35:135A, 1967 (Abst.)

2. Vail, J. M. Relation of energy metabolism to growth and differentiation in L-cell suspension cultures, Doctoral Dissertation, University of Maryland, 1967.

3. McAtee, Lloyd T. and J. R. C. Brown, Cytogenetic and kinetic effects of a sub-lethal heat shock on a heteroploid cell suspension culture. Trans, Amer. Microsc. Soc. 87:122, 1968. (Abst.)

4. McAtee, Lloyd T. and J. R. C. Brown, The inhibition of DNA synthesis in suspension cultures of L-cells by a sub-lethal heat shock. Abstracts, 19th Annual Meeting of the Tissue Culture Association p. 34, 1968.

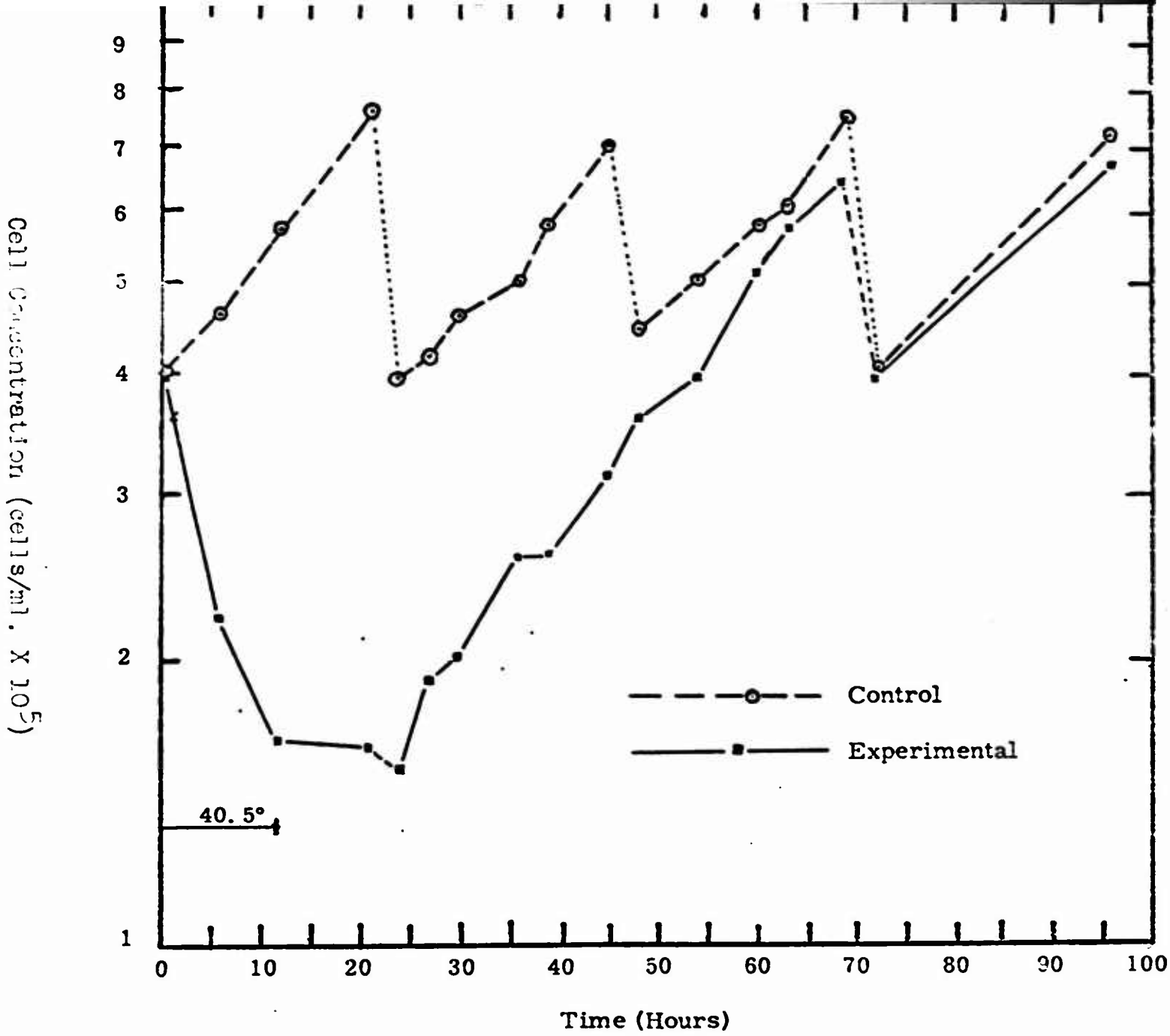

Figure 1. Growth curves for suspension cultures of mouse fibroblast L-929 cells incubated at 35. 5° C with and without a 12-hour heat stress at 40. 5° C.

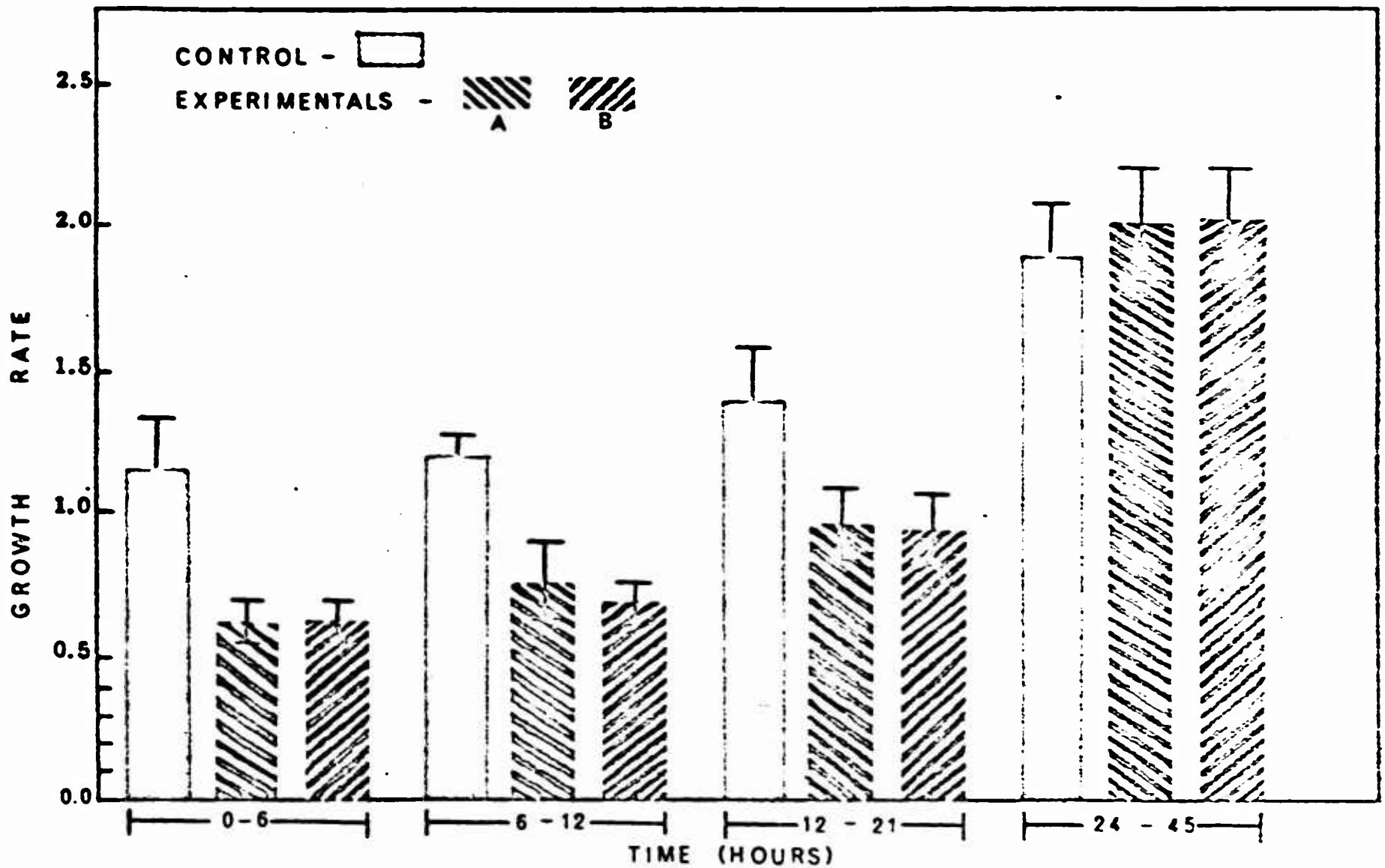

FIGURE 2: THE EFFECT OF A 40.5°C. HEAT SHOCK ON THE GROWTH RATE OF MOUSE FIBROBLASTS IN SUSPENSION CULTURE.

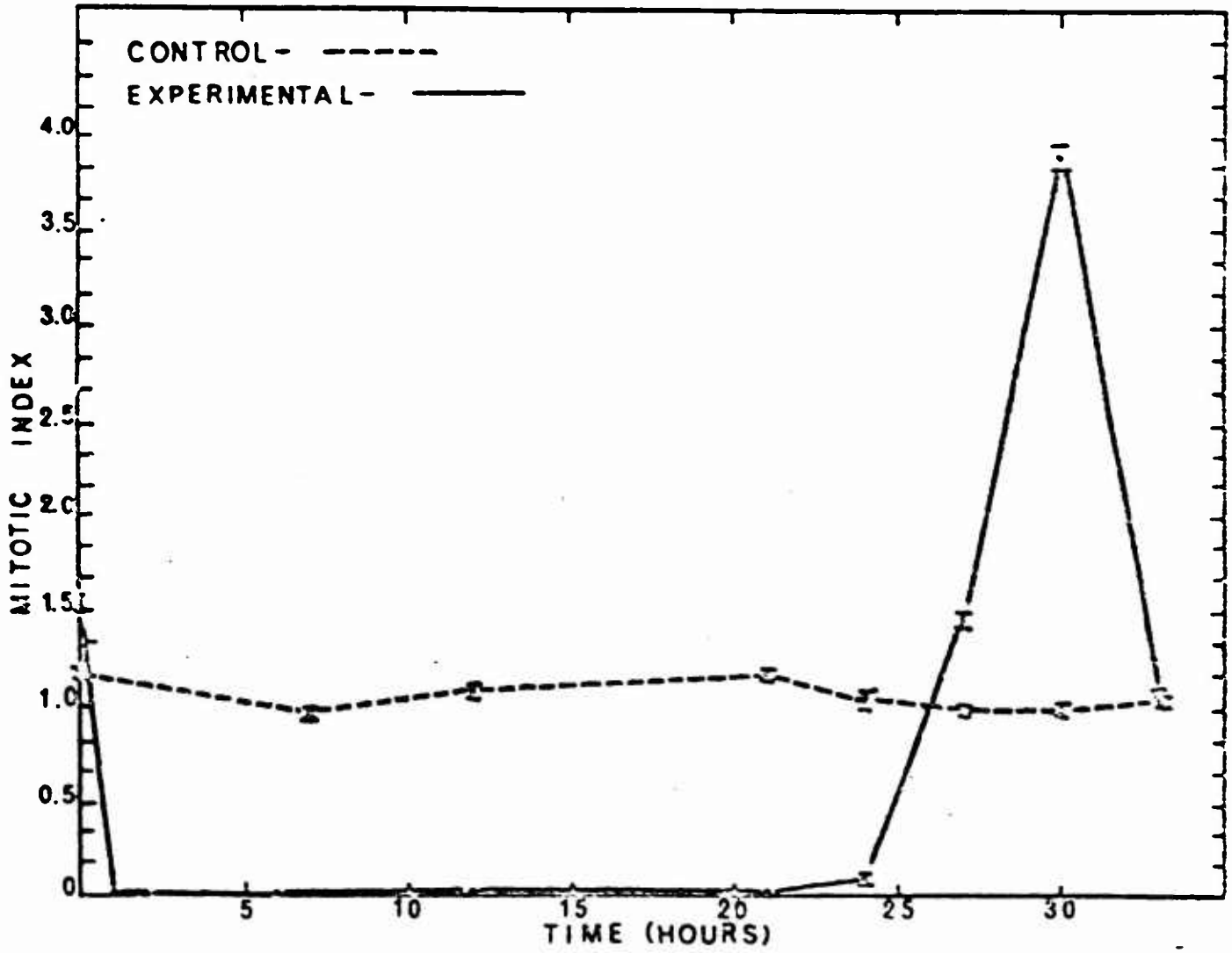

FIGURE 3: THE EFFECT OF A 40.5°C. HEAT SHOCK ON THE MITOTIC INDEX OF MOUSE FIBROBLASTS IN SUSPENSION CULTURE.

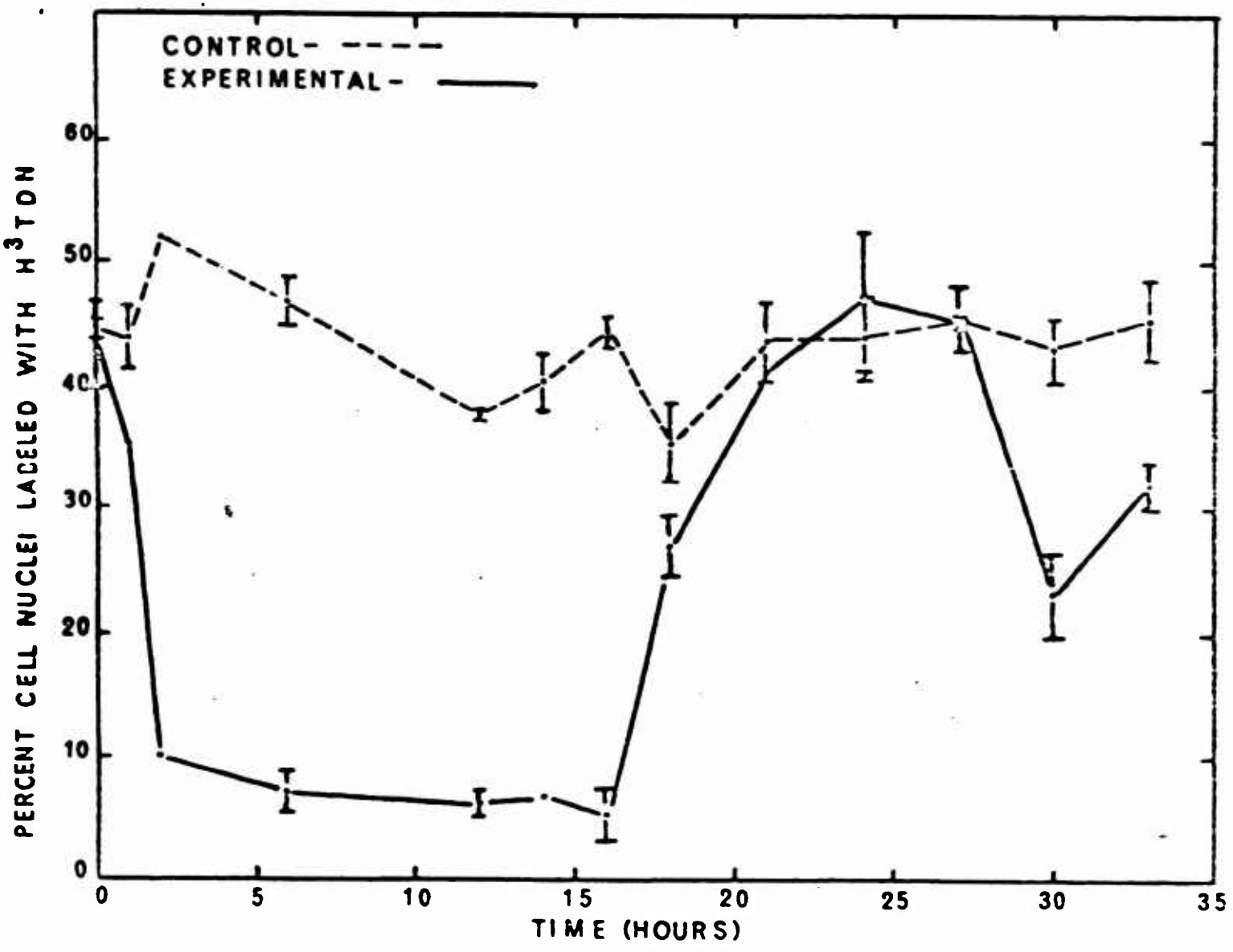

FIGURE 4: THE EFFECT OF A 40.5°C. HEAT SHOCK ON THE DNA SYNTHESIS OF MOUSE FIBROBLASTS IN SUSPENSION CULTURE.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6455	CSCRD-103

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

10a. CURRENT NUMBER/CODE
61130011 3A013001A91C CC 113

10b. PRIOR NUMBER/CODE

11. TITLE (U) EFFECTS OF PHYSICLOGICAL AND PSYCHOLOGICAL STRESSUPON INFECTION AND DISEASE

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 MICROBIOLOGY 003900 CLINICAL MEDICI... 016200 STRESS PHYSICLOGY	10 64	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	PRIOR FY 69	2	82
		CURRENT FY 69	2	75

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21. TECHNOLOGY UTILIZATION
PUBLIC HEALTH

22. COORDINATION
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 PERSONEL, 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.

(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 PATIENT /S 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.

(U) PROGRESS - JUL 67 THRU JUN 68 WITHIN THE PAST YEAR NEWLY DEVELOPED HORMONE ASSAY PROCEDURES, INCLUDING THOSE FOR URINARY TESTOSTERONE, PLASMA GROWTH HORMONE, AND PLASMA THYROTROPIC HORMONE MEASUREMENTS, HAVE BEEN APPLIED TO FROZEN SAMPLES STILL IN STORAGE FROM THIS STUDY. THESE ANALYSES CURRENTLY IN PROGRESS SHOULD BE MOST VALUABLE IN CLARIFYING THE INTERPRETATION OF EARLIER FINDINGS WITH LESS SPECIFIC MEASUREMENTS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. CONSEC 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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 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 (DC&I)

Investigators.

Principal: COL Edward L. Buescher, MC; John W. Mason, M.D.

Associate: CPT Robert M. Rose, MC; CPT Richard O. Poe, MC; CPT Marvin S. Wool, MC; CPT Borden E. Howland, MSC; Edward H. Mougey, M.S.; Frances E. Wherry, A.B.; David R. Collins, B.S.; Elizabeth D. Taylor, M.S.; Percy T. Ticketts, B.S.; Norman Krasnegor, M.S.

Description.

This study was designed to explore the possibility that stress-related, pre-illness changes in hormonal levels may play a contributory role in the pathogenesis of acute respiratory infections. The feasibility of the study was suggested by the high incidence of acute adenovirus infections in Army recruits during basic training in the winter months at Ft. Dix, New Jersey. Furthermore, the great majority of such illnesses usually are clustered during the third and fourth week of basic training. It was, therefore, possible to study a population in which a very high incidence of respiratory illness could be predicted within a designated two-week period.

Progress.

Continuous 24-hour urine samples were collected on an entire platoon of 48 soldiers throughout the basic training period. Urine collection was constantly supervised by a team of technicians (enlisted men) who followed the platoon with the collection bottles during the day and who were stationed in the barracks' latrine during the night. At the end of eight-hour periods, the urine was placed in freezers at -10°C. Daily urine samples were kept frozen on each man for approximately one week or longer so that when signs of clinical illness appeared, sufficient samples were available so that urinary hormone determinations could be made during a substantial pre-illness period. Early morning blood samples were obtained once weekly on each subject.

Each evening oral temperatures were taken and each subject examined and rated on a four-plus scale for signs of pharyngitis, rhinorrhea, cough, or "toxicity." Conventional microbiological techniques for recovery and identification of common bacterial and virological pathogens were employed at frequent intervals.

In this platoon 17 men were hospitalized with moderate or severe acute respiratory infections. Of these, pre-illness urine samples covering

at least a seven-day period were available on 12 men. Several men who were clinically ill but refused hospitalization were also studied. As a control group, 12 men having the lowest cumulative rating of signs of illness for the entire basic training period were chosen for comparison with the most severely ill group.

From an endocrinological standpoint, an attempt was made to obtain as broad a view of endocrine function as possible, on the assumption that the state of host "resistance" is not likely to be a function of any single hormone, but rather a function of the "overall" hormonal balance involving many hormones which may exert interdependent influences on the mechanisms involved in immunity and "resistance." Accordingly, urinary 17-hydroxycorticosteroids (17-OHCS), epinephrine, norepinephrine, estrone, estradiol, estriol, testosterone, androsterone, etiocholanolone, dehydroepiandrosterone, and aldosterone determinations were made. Plasma thyroid hormone determinations were made with the butanol-extractable iodine (BEI) procedure and plasma insulin levels were determined by radioimmunoassay.

It was found that in most cases the onset of fever and symptoms was relatively abrupt and that these signs provided a more reliable index of onset of illness than did the time of hospitalization. Studies of hormone levels during the period of seven days or longer preceding onset of fever revealed several types of pre-illness hormonal changes or abnormalities.

1. Slow and Progressive Hormonal Changes. In over 90% of subjects, thyroid hormone levels (BEI) showed a gradual decrease to the point of onset of illness, followed by a marked elevation one week later. A gradual decrease in estradiol and estriol levels for several days prior to onset of illness was also observed in many subjects.

2. Abrupt "Spiking" Hormonal Changes. In about 75% of subjects, a sudden elevation of urinary 17-OHCS levels occurred about three days prior to the onset of illness. In most cases there was a similar spiking of urinary epinephrine and norepinephrine levels at the same time. In many instances, these abrupt changes in adrenal hormone levels coincided with particular stressful experiences. Similar but less consistent abrupt changes were seen in urinary androgen levels.

3. Sustained Changes in Absolute Hormonal Levels. Mean urinary 17-OHCS levels during the pre-illness week were elevated in comparison with levels of a similar group of control subjects with minimal or no respiratory illness. The data also suggest similar mild or moderate elevations in levels of epinephrine and insulin and lowered levels of estrogens and aldosterone during the pre-illness week. Urine osmolarity, incidentally, was found to be elevated and urine volume was low during the pre-illness week.

Following the definition of the pre-illness changes summarized above, the difficult question of evaluating the possible specificity of any of these changes as pathogenetic factors in the onset of respiratory illness remains, of course, to be considered. Various types of control data are available. Many hormonal values were obtained in the ill subjects during later phases of basic training, following their recovery from respiratory illness, and provide some basis for a longitudinal view of the changes preceding illness. In addition, 12 subjects with minimal illness studied through the same period can be compared in various ways with the subjects who developed illness. An additional group of 48 soldiers was studied in September of the same year, when the incidence of respiratory illness was very low, so that still another population providing some control data is available. At present, various approaches to the analysis of these data are being explored. In addition, during the past year new data from urinary testosterone and plasma growth hormone determinations on frozen samples have been obtained.

In addition to the study of pre-illness hormonal changes, two psychoendocrine studies have emerged from this project. The first was concerned with the multiple determinants of 17-OHCS excretion in recruits during basic training. Recent psychoendocrine studies have shown striking relationships between the effectiveness of psychological defenses and mean excretion of 17-OHCS. The purpose of this study of basic trainees was threefold: (1) to verify these previously-described correlations by using them to predict individual differences in 17-OHCS excretion, (2) to investigate the role of additional psychological, biological, and environmental variables which might be influencing an individual's characteristic adrenocortical level, and (3) to compare individual differences in 17-OHCS excretion with individual differences in the activity of other endocrine systems.

Continuous 72-hour urine collections were made by each of 46 recruits during four consecutive weeks of basic training and analyzed for 17-OHCS. A rating of psychological response to training was assigned to each man on the basis of psychiatric interviews and daily field observations. An automated MMPI was administered to each man by mail after he had completed his six-month tour of active duty.

Correlating individual mean 17-OHCS levels with other biological, psychological, and environmental variables yielded the following results.

1. Body weight was shown to correlate with mean 17-OHCS levels.

2. Psychological predictions based on the estimated effectiveness of a man's psychological defenses correlated with mean 17-OHCS levels.

3. Combining the influence of body weight and psychological ratings predicted mean 17-OHCS levels with greater accuracy than either one alone.

4. The R scale of the MMPI correlated highly with mean 17-OHCS. It was also observed that men whom the MMPI characterized as using the psychological defenses of projection and acting-out tended to fall predominately into the lower 17-OHCS quartiles. The psychological observations made during training suggested that the correlations between MMPI data and 17-OHCS excretion were not necessarily a function of the particular defenses which were employed. It was felt, however, that particular defensive styles or constellations were more or less effective in coping with feelings of being threatened or overwhelmed in the basic training situation.

5. Twelve of fourteen men who had lost a parent by death were found in the upper and lower 17-OHCS quartiles.

6. The 17-OHCS group mean rose from 7.2 mg.% to 8.4 mg.% between the third and fourth weeks, at the same time as the mean environmental temperature fell 23 degrees. That the 17-OHCS change might be a response to distress caused by cold was suggested by the fact that the lightest (and usually thinnest) men had greater elevations in both 17-OHCS and BEI from week three to week four than the heaviest men.

These results suggest that an individual's characteristic level of adrenal cortical activity may be influenced by psychological, biological, historical and environmental factors. Recognizing the possible influence of a variety of different variables on 17-OHCS excretion not only highlights the methodological problems of psychoendocrine research, but also emphasizes the significance of those psychological-adrenal cortical relationships which have emerged.

In a second psychoendocrine study, possible interrelationships between psychological factors and a number of hormones in addition to the corticosteroids were explored. Rather extensive data have been accumulated on the relationship between an individual's chronic level of 17-OHCS excretion and the nature of his psychological interaction with the environment. As yet, relatively little information has been obtained on possible relationships between psychological functioning and estimates of individual's mean or characteristic level of activity in other endocrine systems. This issue was explored in a platoon of healthy men recently recruited into the Army at Ft. Dix. Forty-six men, comprising an entire platoon, had their urine collected over the first month of basic training. Each man had an interview with a psychiatrist and was observed daily by a team of three enlisted men. From this data effectiveness of psychological defenses during training was predicted and found to correlate significantly with the excretion of 17-OHCS. In addition, the determination of a chronic, monthly value for each man of urinary epinephrine, norepinephrine, estrone, estriol, estradiol, testosterone, androsterone, etiocholanolone, and three specific 11 oxo 17-ketosteroids was performed. Weekly measurements of fasting plasma BEI and plasma insulin were also made. Initial analysis of this large body of data has proceeded along three lines.

1. Correlation of each of the other endocrine variables with the individual's 17-OHCS level. This provides some information as to possible relationships with those psychological parameters relating to 17-OHCS excretion as well as indications as to which hormones may parallel the corticoids, reflective of possible integrating mechanisms.

2. Investigation into other variables, such as body weight, that may effect the other hormones studied, similar to what has been found to influence 17-OHCS excretion.

3. Preliminary search for additional psychological characteristics that may correlate with these other hormones studied.

The excretion of epinephrine and norepinephrine was found to relate to several different variables. Men in the highest quartile of 17-OHCS excretion, reflective of diminished effectiveness of their psychological defenses, also excreted significantly more epinephrine over the month studied than those men in the lowest 17-OHCS quartile. The relationship is not an invariable one, however, with several exceptions noted, indicating the probable influence of other variables. It was found that smokers tended to excrete more epinephrine, less norepinephrine, and showed a significantly higher epinephrine-norepinephrine ratio than nonsmokers. The possible influence of differing ways of handling anger upon epinephrine-norepinephrine excretion, as described by Funkenstein, was investigated in these recruits. Two groups of nine men each were selected, without knowledge of the catecholamine data, to represent the extremes of "anger in" and "anger out" behavior. Consistent with the hypothesis, "anger in" men excreted significantly more epinephrine than those in the "anger out" group. A rating of motor activity on a one to seven scale was given to each recruit by three observers independently. No correlations were observed between these ratings and the excretion of epinephrine and norepinephrine.

Fasting plasma insulin, determined by radioimmunoassay, was found to correlate very highly with 17-OHCS excretion, $r = .87$, $n = 24$. Although individuals tend to fluctuate over time, the mean insulin level for the men in the highest 17-OHCS quartile was 12.4 ± 6.3 uu, as opposed to 1.1 ± 1.2 uu, for those in the lowest quartile, with each individual characterized by a mean of four or five weekly measurements. Insulin correlated positively with body weight but also was found to correlate significantly with weight-corrected 17-OHCS values and psychological ratings. This would indicate that the insulin - 17-OHCS relationship exceeded that due to body weight alone.

The mean BEI values all fell within a euthyroid and quite restricted range, 2.5 ug.% to 4.9 ug.%. The mean BEI level for 30 out of 46 men fell within a one microgram range, 3.5 to 4.5 ug.%. This reflects the small degree of variation often seen in this measurement. BEI values were not observed to correlate directly with 17-OHCS, psychological ratings, or ratings of motor activity.

Both the values for estrone and for the total of estrone, estradiol and estriol excreted were found to correlate with psychological ratings. These estrogens, however, were not significantly correlated with 17-OHCS excretion. This suggests that the psychological ratings were correlated with levels of 17-OHCS and estrogens excreted, but that individuals did not necessarily have to demonstrate parallel responses in both these endocrine systems.

The various androgens measured demonstrated no direct association with either 17-OHCS values or with psychological ratings described. It was noted, however, that androsterone and etiocholanolone and their 11-oxo forms, 11-hydroxyandrosterone, 11-hydroxyetiocholanolone, and 11-keto-etiocholanolone did not always correspond with one another. These metabolites comprise the major portion of the urinary 17-ketosteroids. As dissociations were frequently observed among the various components of this class, the value of the overall 17-ketosteroid measurement can be questioned.

It becomes apparent in the analysis of the data collected that the search for possible psychoendocrine relationships not only rests on careful evaluation of individual differences in psychological responses to the environment, but that the influence of a variety of other variables must be carefully evaluated. As the search for these psychoendocrine relationships is a major continuing goal of our group, the results presented must be regarded as preliminary and hopefully will lead to more specific hypotheses to test.

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 (OFF / WHD)	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
30 06 68	C. TERMINATED 01 07 67	U / U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 117

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) PROBLEMS IN MATRIX THEORY

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012100 ORGANIC CHEMISTRY 009700 MATHEMATICS AND S	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. NUMBER DA 49 193 MD 67 6709246	PRIOR FY 67	0	4
	c. TYPE Y.GRANT d. AMOUNT $5,825	CURRENT FY 68	0	6

19. GOVT. LAB/INSTALLATION/ACTIVITY

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INVESTIGATORS PRINCIPAL: PEARL, M. H.
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21. TECHNOLOGY UTILIZATION: MATRIX THEORY

22. COORDINATION: NA

23. KEYWORDS: MATRIX THEORY, ALGEBRA, MATHEMATICS.

24.

(U) TECH OBJECTIVE - DR. PEARL IS STUDYING THE PROPERTIES OF GENERALIZED INVERSES OF MATRICES. THE UNDERSTANDIAG OF THESE MATRICES IS ESSENTIAL FOR THE PROPER EVALUATION OF SUBSTITUENTS ON EITHER PROPOSED OR SYNTHESIZED CHEMICAL MOLECULES.

(U) APPROACH- (1) TO FIND THE NECESSARY AND SUFFICIENT CONDITIONS UNDER WHICH (AB) EQLALS (B)(A). (2) TO OBTAIN A GENERALIZED INVERSE OF THE SQUARE SINGULAR MATRIX BY MEANS OF A LIMITING PROCESS BEGINNING WITH A NON-SINGULAR MATRIX. (3) EXTEND THE CONCEPT OF A GENERALIZED INVERSE TO A MATRIX WITH ENTRIES FROM AN ARBITRARY FIELD. DR. PEARL ALSO PLANS TO STUDY EPR AND NORMAL EPR MATRICES.

(U) PROGRESS - JUL 67 THRU JUN 68 THE INVESTIGATION OF THE STRUCTURE OF THE GENERALIZED INVERSE(S) OF MATRICES WITH ELEMENTS FROM (I) EITHER THE REAL OR COMPLEX FIELDS, AND, (II) AN ARBITRARY FIELD HAS BEEN CARRIED FORWARD DURING THE PAST YEAR. EACH OF THE FOUR IMPORTANT TYPES OF GENERALIZED INVERSES FOR MATRICES WITH ENTRIES FROM AN ARBITRARY FIELD HAS BEEN STUDIED IN DETAIL. (FOR THE REAL COMPLEX FIELDS, THESE INVERSES ARE IDENTICAL AND REDUCE TO THE USUAL GENERALIZED INVERSE.) IN PARTICULAR, NECESSARY AND SUFFICIENT CONDITIONS WERE FOUND FOR THE EXISTENCE OF EACH OF THE FOUR TYPES. AN EXPLICIT EXPRESSION WAS FOUND FOR THOSE GENERALIZED INVERSES WHICH EXIST AND SEVERAL RELATIONSHIPS BETWEEN THE VARIOUS GENERALIZED INVERSES WAS FOUND. IN SUBSEQUENT WORK, AN INVESTIGATION WAS BEGUN OF THE GENERALIZED INVERSES OF SPECIAL MATRICES (E.G. NORMAL, SYMMETRIC, ETC.) THIS WORK UNIT WAS TERMINATED 30 JUN 68. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

27. COMMUNICATIONS SECURITY	29.	29. OSD CODE	30. BUDGET CODE
☐ 2. COMSEC OR COMSEC RELATED · ☒ 3. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In Thousands)	36.

DD FORM 1 JAN 65 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 117, Problems in matrix theory

Investigator.
Principal: Martin H. Pearl

Description.

The purpose of this work is to study some of the properties of generalized inverses of matrices. It is possible to divide the substituents of a given set of chemical molecules into components which can then be given a value depending upon the numerical result obtained using that molecule in a specific test system. The purpose of such a procedure is to cut down on the synthesis of the number of analogs required to develop the most active one in any given limited set.

Progress.

The investigation on the structure of the generalized inverse(s) of matrices with elements from (i) either the real or complex fields, and (ii) an arbitrary field has been carried forward during the past year. Each of the four important types of generalized inverses for matrices with entries from an arbitrary field has been studied in detail. (For the real and complex fields, these inverses are identical and reduce to the usual generalized inverse). In particular, necessary and sufficient conditions were found for the existence of each of the four types. An explicit expression was found for those generalized inverses which exist and several relationships between the various generalized inverses were found. In subsequent work an investigation was begun of the generalized inverses of special matrices (e.g., normal, symmetric, etc.).

Summary and Conclusions.

Dr. Pearl feels that the techniques now available within the Division of Medicinal Chemistry are sufficiently well developed that they can be applied directly to answer the problem. He further feels that some of the techniques which have been borrowed by the Division outside of the Institute are also sound. It is not yet certain whether direct application will be made of the generalized solutions which Dr. Pearl has worked on.

Publications.

Pearl, M. H. On Generalized Inverses of Matrices. Proc. Cambr. Philos. Soc., 1967.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6500	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY (a. CLAS. b. DOWN.)	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
31 05 68	C. TERMINATED 01 07 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 118

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) ELECTRON MICROSCOPY OF INTESTINAL EPITHELIUM

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CMPL. DATE	15. FUNDING AGENCY
003500 CLINICAL MEDICINE	02 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
B. CONTRACT	a. NUMBER DA 49 193 MC 2705	PRIOR FY 67	2	18
	b. TYPE A.FPF d. AMOUNT $44,390	CURRENT FY 68	0	0

19. GOVT. LAB/INSTALLATION/ACTIVITY
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ASSOCIATE: CONRAD, LTC M. E.
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21. TECHNOLOGY UTILIZATION: MEDICAL RESEARCH

22. COORDINATION: NA

23. KEYWORDS: HEPATITIS, SPRUE, ELECTRON MICROSCOPE, VIRUS, INTESTINE.

24.

(U) TECH OBJECTIVE - TO CHARACTERIZE THE INTESTINAL LESIONS OF TROPICAL SPRUE AND INFECTIOUS HEPATITIS. TO ATTEMPT TO IDENTIFY VIRUS PARTICLES IN SPECIMENS FROM PATIENTS WITH THESE DISEASES.

(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 PHYSICLOGIC ABNORMALITIES AND ATTEMPT TO DEMONSTRATE VIRAL PARTICLES.

25.

(U) PROGRESS - JUL 67 THRU JUN 68 DURING THE TERMINAL YEAR OF THE CONTRACT ADDITIONAL OBSERVATIONS WERE MADE OF ELECTRON MICROSCOPIC SECTIONS OF INTESTINAL SPECIMENS FROM PATIENTS WITH ACUTE INFECTIOUS HEPATITIS. MULTIPLE STELLATE SHAPED PARTICLES WERE OBSERVED WITHIN THE CYTOPLASM OF DUODENAL AND JEJUNAL EPITHELIAL CELLS. THESE PARTICLES SEEMED TO BE AGGREGATES OF 8 TO 12, 18 TO 20 MILLIMICRON PARTICLES. UNIFORMITY SEEMED TO INDICATE THEY WERE NOT GLYCOGEN. LACK OF A CRYSTALLINE ARRAY MADE IT IMPOSSIBLE TO INSURE THAT THE PARTICLES OBSERVED IN THESE PREPARATIONS WAS A VIRUS AND NOT AN UNUSUAL NUMBER OF POLYRIBOSOMES. SPECIAL PREPARATIONS OF TISSUES WILL BE REQUIRED FOR FURTHER IDENTIFICATION. FUNDING OF CONTRACT IS TERMINATED BECAUSE OF UNAVAILABILITY OF THESE MATERIALS AT PRESENT. INVESTIGATORS WILL CONTINUE INVESTIGATION OF EXISTANT SPECIMENS DURING TERMINAL YEAR WITHOUT ADDITIONAL FUNDING. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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)	35.

DD FORM 1 JAN 65 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C

Task 01, In-House Laboratory Independent Research

Work Unit 118, Electron microscopy of intestinal epithelium

Investigators
Principal: Roberta Hartman, PhD
Associate: Richard Hartman, PhD and LTC M. E. Conrad, MC

Description.

A collaborative project to investigate the electron microscopic changes in intestinal biopsy specimens from patients with infectious hepatitis.

Progress.

During 1962 and 1964 small intestinal biopsy specimens were obtained from soldiers with documented infectious hepatitis in Korea. Examination of these biopsy specimens by light microscopy showed marked changes of the epithelium, lamina propria and submucosa during the acute stage of illness. The repeated failure of many investigators to demonstrate viral particles in electron microscopic sections of liver from patients with infectious hepatitis, and the frequent occurrence of intestinal lesions during the acute stage of illness, led us to postulate that intestinal cells might be replete with virus particles while other tissues contained few viral particles. Thus, hepatitis might be similar to poliomyelitis and frequently involves the gut but only occasionally causes injury to other organs. To investigate this hypothesis multiple small intestinal biopsy specimens of duodenum and jejunum were prepared for electron microscopic examination. During 1965-6, studies of these sections showed many 200 Angstrom particles in the cytoplasm of jejunal epithelial cells which frequently aggregated to form rosettes with a stellate configuration. The failure to find these particles in a crystalline like array made it impossible to differentiate them easily from certain cellular organelles. Preliminary studies were initiated with a microdensitometer to distinguish these particles from organelles such as ribosomes which they most closely resemble. Further examination of additional sections should be performed in an attempt to delineate better the identified inclusion bodies within intestinal cells and ascertain if they are viral particles. Progress has been halted during the last year because of the unavailability of a functioning electron microscope. Planned studies during the next fiscal year will be performed without additional funds.

Summary and Conclusions

Small particles have been identified by electron microscopy in the cytoplasm of specimens of small intestinal epithelium from patients with infectious hepatitis but not in similar specimens obtained from normal subjects. The possibility that they represent an etiologic virus causing hepatitis is considered. Attempts to distinguish these abnormal particles from other intracellular inclusion bodies are the present purpose of this study.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6501	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY PRT	WHS	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE C1 01 68	U	U	NA	GA	A. WORK UNIT

10. CURRENT NUMBER/CODE
61130011 3A013001A91C 00 119

10.1 PRIOR NUMBER/CODE

11. TITLE (U) CYTOCHEMICAL ANALYSIS OF GROWTH OF MALARIAL PARASITES 09

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012300 BIOCHEMISTRY 010100 MICROBIOLOGY	06 65	NA	OTHER DA

16. PROGRAM METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (in thousands)
	a. NUMBER NA	c. DATE				
C. IN-HOUSE	b. TYPE NA	d. AMOUNT NA		PRIOR FY 68	1	56
				CURRENT FY 69	1	43

19. GOVT. LAB INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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RESP. INDIV. MERONEY, COL W. H. 202-576-3551	INVESTIGATORS PRINCIPAL BAER, G. F. ASSOCIATE STEYN, CPT K. 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.

(U) APPROACH- TO EXAMINE THE CELLULAR AND SUBCELLAR ENTITIES COMPRISING MALARIA PARASITES BY CYTOSPECTROPHOTOMETRY, INTERFERENCE MICROSCOPY AND QUANTITATIVE ELECTRON MICROSCOPY.

(U) PROGRESS - JAN 68 THRU JUN 68 IN EXPERIMENTAL MALARIA RBC CONCENTRATION VARIES INVERSELY WITH PARASITEMIA, THE NORMAL CELL VOLUME MAY BE CHANGED, AND THE HEMATOCRIT DECREASED. HOWEVER, THE EXTENT OF THESE CHANGES IS RELATED TO THE SPECIES OF PLASMODIUM INVOLVED. FOR EXAMPLE, WITH P. CHABAUDI AND P. VINCKEI IN MICE, A PARASITEMIA OF 70-80 PERCENT WITH THE FORMER LOWERS THE CELL COUNT BY 40 PERCENT, WHILE FOR THE LATTER A PARASITEMIA OF 85-95 PERCENT IS REQUIRED FOR A COMPARABLE DEPRESSION. P. CHABAUDI ALSO DECREASES THE HEMATOCRIT EARLIER THAN P. VINCKEI. WITH P. CHABAUDI RBC VOLUME CHANGES ARE EVIDENT IN PARASITEMIAS OF 70-75 PERCENT AS COMPARED WITH AN 85 PERCENT PARASITEMIA FOR P. VINCKEI. HOWEVER, THE LATTER INCREASES THE MEDIAN VOLUME OF RBCS BY 50 PERCENT WHILE THE LIMIT IS ABOUT 70 PERCENT WITH THE LATTER. FROM THE USE OF SPECIFIC FLUOROCHROMES FOR CYTOCHEMICAL STUDIES AND ELECTRON MICROSCOPY OF CHROMATIN FIBRILS IT HAS BEEN CONCLUDED THAT THE CYTOCHEMICAL MAKEUP OF PLASMODIAL NUCLEI AND STRUCTURAL ORGANIZATION OF THE NUCLEO-PROTEIN DO NOT DIFFER FROM OTHER PROTOZOAN NUCLEI AND HOST CHROMATIN. ALL EFFORTS TO OBTAIN PLASMODIA ENTIRELY FREE FROM HOST CELL MATERIAL HAVE FAILED AND ANY BIOCHEMICAL INVESTIGATIONS WILL HAVE TO TAKE THIS FACT INTO CONSIDERATION. A EUCHRYSINE STAIN HAS BEEN HIGHLY EFFECTIVE FOR DETECTION OF LOW PARASITEMIA BY A FLUORESCENCE TECHNIQUE AND SUGGESTS THE POSSIBILITY OF AN AUTOMATED SLIDE SCANNER. WORK ON QUANTITATIVE ELECTRON MICROSCOPY HAS CONTINUED DEVELOPMENT OF A NEW HIGH VACUUM ELECTRON IRRADIATION CHAMBER INCORPORATING AN INFRARED MICROSCOPE CAPABLE OF RESOLVING 15 MU SPOTS AND A SMALL MASS SPECTROMETER FOR ANALYSIS OF RELEASED GASES FOR MASSES UP TO 200. THE SIGNIFICANCE OF THIS WORK RESIDES IN THE PROSPECT OF REACHING RESOLUTIONS OF 1 ANGSTROM UNIT IN ELECTRON MICROSCOPES. PHENYLALANINE HAS BEEN USED AS A BASIC MODEL FOR PROTEIN. IRRADIATED SAMPLES HAVE BEEN CHROMATOGRAPHED AND IDENTIFICATION OF IRRADIATION PRODUCTS IS IN PROGRESS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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. PROCESSING AGENCY	34. SPECIAL EQUIPMENT
EST. FUNDS (in thousands)	35.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 119, Cytochemical Analysis of Growth of Malarial Parasites

Investigators.
Principal: G.F. Bahr, M.D.
Associate: Kurt Stenn, CPT, USAF (MC)

Description.

This is a multifaceted study with three primary objectives. These are: (1) to determine the effect of the plasmodium on the host red cell in terms of volume, osmotic fragility, electrophoretic mobility and dry mass by a series of biophysical and cytochemical analyses of the parasite throughout its erythrocytic life cycle; (2) to perform a feasibility study on the design and construction of a rugged optical scanner for blood smears capable of detecting and determining a parasite/white cell ratio; and (3) to continue studies dealing with the multiparameter analysis of electron beam-object interactions in the electron microscope.

Progress.

Studies of red cell volume, concentration and hematocrit in mice were continued with the aid of a Coulter counter manometer, fitted with a custom made aperture and linked to a 400 channel analyzer system with a punched paper printout.

A blood diluent consisting of a buffered and supplemented saline (Isotone) was used to which 0.25% irradiated human serum was added. Cell sizes remain constant for one half hour in this solution.

If parasitemia in percent is compared with red cell count, there is a constant decrease in red cell concentration with increasing infection. The extent of this change appears to be related to the species of plasmodium involved. For example, with P. chabaudi and P. vinckei in mice, a parasitemia of 70-80% with the former depresses the cell count by 40% while a parasitemia of 85-95% with P. vinckei is required for the same depression.

Volume changes of erythrocytes as a consequence of infection with P. vinckei and P. chabaudi become noticeable only at rather high levels of parasitemia. For the former parasite this level is as high as 85% while for the latter one it is somewhat lower, or about 70-75%. Also, the absolute values of volume changes

differ significantly between these parasites: P. vinckei increases the median volume of red cells by up to 90%; whereas in P. chabaudi infection no value higher than about 70% was found.

When parasitemia is compared to the hematocrit, there is a slow decrease of the hematocrit from a normal of 43% to about 32% when infection involves more than 70% of the red cells. Infected mice have hematocrits as low as 10% in terminal stages. Again, P. chabaudi affects the hematocrit at an earlier time than P. vinckei.

In a continuation of cytochemical investigations of the composition of the plasmodial nucleus the use of the fluorochrome Brilliant Sulfaflavine has permitted fluorometric comparisons of arginine/DNA ratios in mouse thymus lymphocytes and plasmodia. The results indicate that this ratio in parasite nuclei of all stages is closely comparable to the model of a mammalian cell used as test.

Lysine can also be labeled with a rather specific fluorochrome. This is Dansylchloride, or 1-dimethylaminonaphthalene-5-sulfochloride which in an ethanol medium is essentially specific for lysine in protein bound amino acids. Experiments have shown that parasite nuclei are positive for lysine.

These and earlier observations lead to the conclusion that the cytochemical makeup of the plasmodial nucleus is comparable to that of other protozoan and to mammalian cells. In addition, electron microscopy of parasite chromatin fibrils has shown that the structural organization of the nucleoprotein is also comparable to that of other protozoan and to host chromatin. This suggests that monocellular models for the study of the interactions of antimalarials with nuclear genetic mechanisms on the molecular level should be protozoans or mammalian tissue culture cells.

Despite much effort all attempts to isolate truly "clean" parasites entirely free from any remnants of host cell material have met with failure. Whenever a good effect on the erythrocyte was observed the damage to the parasite was extensive. Therefore, biochemical investigations will have to take this fact into consideration.

A Euchrysine stain has been shown to be very useful in the detection of plasmodia by fluorescence in low parasitemias. In each instance when Giemsa staining was compared to the fluorescence technique the latter has proved superior. Furthermore, the use of phase contrast in transmission microscopy has been successfully joined with the excitation of fluorescence by incident illumination, combining the visualization of the

non-lysed red cell smear with the sensitivity of detecting parasites by fluorescence. This has lead to a proposal for a feasibility study on the design and development of an automated slide scanner.

Present work on the electron irradiation study consists of two phases: (1) The construction of a new high-vacuum electron irradiation chamber containing facilities for analyzing the visible and ultraviolet fluorescence of specimens during bombardment; and (2) the use of an infrared microscope looking at the specimen during irradiation, capable of resolving 15 mu spots and thus temperature gradients in specimen and object carrier. Furthermore, a small mass spectrometer will analyze the released gases for masses up to 200.

Using phenylalanine, its dipeptide and polypeptides as a basic model for protein, its mass loss and molecules emanating during irradiation have been recorded. The irradiated samples are being chromatographed after various electron doses, and the irradiation products are in the process of being identified. The significance of these studies resides in the prospect of reaching resolutions of 1 Angstrom unit in electron microscopes, i.e., with the prospect of depicting molecular structures.

There has been further development of the technology of quantitative technique for highly sensitive dry mass determinations. Instrumentation for use of 70 mm film in electron microscopes and on quantitative photometers has been completed. It is now possible to photograph up to 48 fields in the electron microscope without reloading and then processing this strip rapidly on the photometer. The latter is now interfaced with an electronic data logging system, which permits the large number of measurements to be stored on magnetic tape for subsequent processing on an IBM 360/30 computer. Likewise, data from the fluorometer will be given through the data logging system to tape for computer analysis. This will permit for the first time quantitative assay of characteristics of the fluorescent decay curves, which appear on the oscilloscope at least to differ significantly at various stages of parasite development.

Summary and Conclusions.

a. Precise data have been obtained on changes in volume hematocrit and cell concentration of infected erythrocytes in P. chabaudi and P. vinckei infections in mice. The extent of these changes has been found to be related to the species of plasmodium involved.

b. Cytochemical and electron microscopy studies of plasmodial nuclei indicate that the composition of the nucleus and the

structural organization of the nucleoprotein are comparable in all respects to nuclei of other protozoans and to mammalian cells.

c. Fluorescence microscopy using a Euchrysine stain has been shown to be superior to Giemsa staining for detection of low parasitemias. This has suggested the feasibility of an automated slide scanning device for use in the field.

d. Equipment has been devised and is being tested which may permit reaching resolutions of 1 Angstrom unit in electron microscopes.

e. Further development of the technology of the quantitative technique for highly sensitive dry mass determinations, plus coupling to the computer for analysis, will permit quantitative assay of fluorescent decay curve characteristics which appear to differ significantly at various stages of parasite development.

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6505	CSGRD-103

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

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 120

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) BEHAVIORAL BASELINES FOR THE EXPERIMENTAL STUDY OF UREMIA

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
003500 CLINICAL MEDICINE 012900 PHYSIOLOGY 016200 STRESS PHYSIOLOGY	11 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
B. CONTRACT	a. NUMBER: DA 49 193 MC 2819 b. TYPE: N.CPFF d. AMOUNT: $82,910	PRIOR FY 68	1	33
		CURRENT FY 69	1	32

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INVESTIGATORS PRINCIPAL: FINDLEY, DR J D
ASSOCIATE: TESCHAN, LT COL P E
TEL.: 301-587-2909 TYPE: UA

21. TECHNOLOGY UTILIZATION: ARTIFICIAL KIDNEY DESIGN

22. COORDINATION: NA

23. KEYWORDS: KIDNEY, BODY FLUIDS, HEMODIALYSIS, ANURIA, UREMIA.

24.

(U) TECH OBJECTIVE - TO ELUCIDATE THE PATHOGENESIS OF THE UREMIC SYNDROME, TO IDENTIFY CHEMICAL FACTORS PRESUMABLY RESPONSIBLE FOR UREMIC SYMPTOMATOLOGY.

(U) APPROACH- UREMIA IS RECOGNIZED AS AN IDENTIFIABLE CONSTELLATION OF ABNORMAL BEHAVIORAL PHENOMENA. QUANTITATIVE TECHNICS OF EXPERIMENTAL PSYCHOLOGY ARE EMPLOYED IN OPERANT-CONDITIONED PRIMATES (RHESUS MONKEYS) TO TRACE BEHAVIORAL DECREMENTS (1) IN THE COURSE OF ACUTE AND CHRONIC RENAL FAILURE AND (2) IN RESPONSE TO INFUSION OF DISCRETE CHEMICAL SUBSTANCES OR OF MATERIALS DERIVED FROM UREMIC PATIENTS. SELECTED SUBJECTS ARE ALSO STUDIED BY MEANS OF ELECTROENCEPHALOGRAMS.

(U) PROGRESS - JUL 67 THRU JUN 68 SUBHUMAN PRIMATES (RHESUS MONKEYS) HAVE BEEN TRAINED TO PERFORM SEVERAL TASKS BY LEVER PRESSING (SHOCK AVOIDANCE WITH TIME PERIOD DISCRIMINATION, FOOD REWARD, TONE COUNTING). UREMIA WAS PRODUCED BY BILATERAL URETERAL LIGATION. DURING THE COURSE OF THE UREMIC PERIOD, SERIAL OBSERVATIONS WERE MADE OF BEHAVIORAL TRAINING PERFORMANCE, BLOOD VALUES OF PH, HEMATOCRIT, TOTAL SOLIDS, UREA, CREATININE, SODIUM, POTASSIUM AND OSMOLALITY WERE MEASURED, AND ELECTROENCEPHALOGRAPHIC RECORDINGS WERE MADE. INITIAL EXPERIENCE REVEALED THAT THERE WERE SOME EARLY CHANGES IN THE ELECTROENCEPHALOGRAPHIC RECORDINGS AND FOOD RESPONSES DROPPED ALMOST IMMEDIATELY, BUT BREAKDOWN IN SHOCK AVOIDANCE WITH TIME PERIOD DISCRIMINATION OCCURRED LATE IN THE COURSE OF THE UREMIA AND WAS NOT CONSISTENT. THE TONE COUNTING PROGRAM, IN WHICH RECENT MEMORY IS TESTED BY THE ABILITY TO COUNT RANDOM TONES, IS PRESENTLY BEING DEVELOPED. A PRELIMINARY STUDY SHOWED THAT SUCH CHANGES IN PERFORMANCE WERE OBSERVED EARLIER IN THE UREMIC PERIOD THAN IN THE SHOCK AVOIDANCE EXPERIMENTS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC or COMSEC RELATED ☒ b. NOT RELATED		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In Thousands)	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 120, Behavioral base lines for the experimental study of uremia

Investigators.
Principal: COL Paul E. Teschan, MC
Associate: Dr. J. D. Findley; CPT Richard M. Finkel, MC; CPT Paul B. Lamborn, Jr., VC; CPT Lionel U. Mailloux, MC; G. J. McCormick, Ph.D.; CPT Coy D. Fitch, MC

Description.

This study attempts to define uremia as abnormal behavior, by means of experimental behavioral techniques. When defined by the sequence of measurable behavioral changes following nephrectomy or ureteral ligation the system may be used to detect and help identify toxic materials removed from uremic humans by dialysis procedures, and to determine the toxicity of specific compounds or body compositional changes. Use of whole organism (primate) behavior affords test criteria which continue in direct relevance to uremia, a feature which is beyond the capability of systems involving lower levels of biological organization.

Progress.

Technics and sequences of surgery, cannulation for chronic infusion and blood sampling, EEG recording, and of systematic data analysis were standardized and proved successful. Initial experiences with peritoneal dialysis including chronic peritoneal cannulation for long-term maintenance of renoprival animals were promising and may permit repeated bioassay procedures in the same trained preparation. In four replications employing a shock-avoidance program of optional-time (early or late) lever-press response, ureteral ligation shifted the alternating or cyclical response pattern toward later responses and progressively-increasing shock-avoidance failure as blood urea nitrogen (BUN) exceeded 200 mg% and uremia became clinically evident. This behavioral program was therefore rejected as being too insensitive, in favor of a program requiring counting. The initial experiment with this program revealed progressive behavioral decrements as BUN approximated 100 mg%, well before clinical uremia could be detected.

Summary and Conclusions.

A behavioral program has been developed which appears to be exquisitely sensitive to toxemic effects of early uremia. If substantiated in replicate experiments the bioassay procedures may be carried forward, combined at a suitable occasion with maintenance or acute dialysis procedures.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION DA CA6SC7	REPORT CONTROL SYMBOL CSCRD-103
4. DATE OF RESUME 01 10 67	5. KIND OF RESUME C. TERMINATED 01 07 67	6. SECURITY a. CNT U b. WHD U	7. REGRADING NA	8. RELEASE LIMITATION GA	9. LEVEL OF RESUME A. WORK UNIT

10a. CURRENT NUMBER/CODE 61130011 3A013001A91C 00 121

10b. PRIOR NUMBER/CODE

11. TITLE (U) SYNTHESIS OF AMINOPHOSPHONATES AS ENZYME INHIBITORS 21

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012600 PHARMACOLOGY 012100 ORGANIC CHEMISTRY 002300 BIOCHEMISTRY	10 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	10 65			
	DA 49 193 MC 2641	PRIOR FY 67	2	20
B. CONTRACT	a. NUMBER J.C c. AMOUNT $40,756	CURRENT FY 68	0	5

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21. TECHNOLOGY UTILIZATION MEDICAL THERAPEUTICS

22. COORDINATION NA

23. KEYWORDS ORGANO-PHOSPHATE, ENZYME INHIBITORS, ALLERGY, COMPLEMENT.

24.

(U) TECH OBJECTIVE - THE OBJECTIVE OF THE WORK IS THE SYNTHESIS OF HERETOFORE UNKNOWN AMINO ALKYLPHOSPHONATES. WHEN SYNTHESIZED THESE COMPOUNDS WILL BE TESTED FOR THEIR ABILITY TO SPECIFICALLY AND IRREVERSIBLY INHIBIT IN VITRO ENZYME SYSTEMS KNOWN OR SUSPECTED TO BE INVOLVED IN ALLERGIC REACTIONS IN VIVO.

(U) APPROACH- THE SNYTHESIS OF COMPOUNDS, SUCH AS PARANITROPHENYL ETHYL AMINO ALKYLPHOSPHONATES WHICH CONTAIN BOTH A REACTIVE NUCLEOPHILE, AND A REACTIVE ELECTROPHILE IN THE SAME MOLECULE PRESENTS A MAJOR PROBLEM. MANY CONDITIONS THAT WOULD INTRODUCE OR UNMASK THE AMINO GROUP WOULD PROMOTE HYDROLYSIS OR CONDENSATION OF THE HIGHLY REACTIVE P-NITROPHENYL GROUP. THE APPROACH WILL BE TO USE A BLOCKING GROUP FOR THE AMINO FUNCTION THE N-TERT. ALKOXYCARBOXYL GROUP WHICH CAN BE REMOVED FROM THE AMINE AFTER THE LABILE ESTER GROUP HAS BEEN ATTACHED UNDER CONDITIONS WHICH WILL NOT REMOVE THE ESTER LINKAGE.

(U) PROGRESS - JUL 67 THRU SEP 67 THE APPROACH OUTLINED ABOVE WAS NOT SUCCESSFUL. THEREFORE THE CONTRACT WAS TERMINATED AS OF 30 SEPT 1967.

26.

27. COMMUNICATIONS SECURITY ☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED	28.	29. OSD CODE BR	30. BUDGET CODE 1
31. MISSION OBJECTIVE NA		32. PARTICIPATION NA	
33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT		
35. EST. FUNDS (In thousands)	36.		

DD FORM 1 JAN 65 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 121, Synthesis of aminophosphonates as enzyme inhibitors

Investigator.
Principal: William J. Bailey, Ph.D.

Description.

The objective of this project was the development of new synthetic methods for the preparation of certain aminoalkylphosphonates. These compounds are of interest as potential specific proteolytic enzyme inhibitors and were to be used for this purpose in connection with other investigations.

Progress.

All efforts to synthesize the required compounds proved to be unsuccessful. The contract was therefore allowed to terminate on its expiration date of 30 Sep 67.

Summary and Conclusions.

None

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6508	CSGRD-102

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U U	NA	0A	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61103011 3A013001A91C 00 122

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) ANTIGENIC AND BIOLOGIC CLASSIFICATION OF DENGUE VIRUSES

12. SCIENTIFIC OR TECH. AREA: 010100 MICROBIOLOGY

13. START DATE	14. EST. COMPL. DATE	15. FUNDING AGENCY
11 65	NA	OTHER: DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	15. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
D. CONTRACT	a. NUMBER DA 49 193 MD 2846 b. TYPE J.C c. AMOUNT $168,808	PRIOR FY 68	3	60
		CURRENT FY 69	1	20

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19. TECHNOLOGY UTILIZATION: VIROLOGY

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ASSOCIATE: HALSTEAD, LTC S.B.
TEL: 203-777-2435 TYPE: UB

22. COORDINATION: NA

23. KEYWORDS: DENGUE, VIRUSES, ANTIGENS, BIOLOGICAL PROPERTIES.

24.

(U) TECH OBJECTIVE - THE ANTIGENIC ANALYSIS OF DENGUE VIRUSES WITH PARTICULAR REFERENCE TO CLASSIFICATION OF WILD VIRUSES RECOVERED IN VARIOUS PARTS OF THE WORLD, CHARACTERIZATION OF BIOLOGICAL PROPERTIES OF WILD DENGUE VIRUSES.

(U) APPROACH- CONVENTIONAL METHODS FOR ANTIGENIC ANALYSIS WILL BE COMPARED WITH NOVEL SYSTEMS EMPLOYING CELL CULTURE TECHNIQUES AND PARTIALLY PURE LINES OF VIRUSES WHEN POSSIBLE. CROSS PROTECTION STUDIES IN RHESUS MONKEYS USING SELECTED WILD DENGUE 1-4 STRAINS WILL BE UNDERTAKEN TO DETERMINE THE DEGREE AND DURATION OF CROSS PROTECTION BETWEEN DENGUE TYPES. LABORATORY RESPONSES TO DENGUE INFECTION WILL BE STUDIED.

(U) PROGRESS - JUN 67 THRU MAY 68 ONE HUNDRED TWELVE MONKEYS OF 4 SPECIES HAVE RECEIVED PRIMARY INFECTIONS WITH WILD STRAINS OF DENGUE 1-4 VIRUSES. OF THESE, 74 HAVE RECEIVED A SECOND SUBCUTANEOUS INOCULATION WITH HOMOLOGOUS OR HETEROLOGOUS DENGUE VIRUS TYPES, 37 HAVE BEEN CHALLENGED WITH A THIRD DENGUE TYPE AND 6 WITH ALL 4 DENGUE VIRUSES. CLINICAL LABORATORY, SEROLOGIC AND VIREMIC RESPONSES TO PRIMARY, SECONDARY AND SUBSEQUENT INFECTIONS ARE BEING STUDIED. IN ANIMALS RECEIVING A SECOND HETEROLOGUS CHALLENGE AT AN INTERVAL OF 6 WEEKS OR 3 MONTHS, THROMBOCYTOPENIA WAS OBSERVED IN 9 RHESUS MONKEYS, MILD HYPOPROTEINEMIA WAS OBSERVED IN 5 AND PROLONGED PROTHROMBIN TIME IN 1 ANIMAL. THE MOST SEVERE RESPONSE OBSERVED WAS IN AN ANIMAL INFECTED WITH DENGUE 4 THEN DENGUE 2 VIRUSES. PRELIMINARY EVALUATION OF CROSS PROTECTION DATA SUGGESTS THAT DENGUE 2 PROVIDES PROTECTION TO INFECTION WITH DENGUE 3 AND 4 VIRUSES, ANY COMBINATION OF 2 SEPARATE DENGUE INFECTIONS PROTECTS AGAINST ANY OTHER TWO. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25. COMMUNICATIONS SECURITY	26.	29. OSD CODE	30. BUDGET CODE
a. COMSEC OR COMSEC RELATED ☐ b. NOT RELATED ☒		BR	1

27. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

EST. FUNDS (In Thousands)	35.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 122, Antigenic and biological classification of dengue viruses.

Investigators.

Principal: Wilbur G. Downs, M.D.

Associate: Scott B. Halstead, Lt. Col. MC, Jordi Casals, M.D.

Description. Two distinct clinical entities have been associated with dengue virus infection: dengue hemorrhagic fever, a syndrome associated with shock, bleeding phenomena and a mortality rate of 5-10% and classic dengue fever, characterized by myalgia, leucopenia, and benign course. When dengue hemorrhagic fever was described in 1956, two new dengue viruses, types 3 and 4, were recovered from cases. In 1958, dengue strains tentatively designated types 5 and 6 were recovered from hemorrhagic fever patients in Thailand. As one explanation for the malignant form of dengue fever, it has been suggested that dengue viruses have acquired virulence properties and that virulence is associated with surface antigen, specifically with dengue types which appear to differ from older prototypes.

It is the purpose of this investigation to study the antigenic and biologic properties of dengue viruses recovered from dengue fever and hemorrhagic fever from several geographical locations. By using an LLC-MK2 plaque reduction neutralization test and single lots of prototype antiserum, attempts will be made to determine whether antigenic variation exists among low mouse passage "wild" dengue strains which would warrant type designations other than types 1-4. Should distinct types or subtypes be discovered an analysis of distribution of strain characters by geographic origin and disease association will be made. Attempts will

also be made to determine whether natural virus populations are composed of mixtures of antigenically variant strains and whether antigenic characters are influenced by host passage. In addition to antigenic analysis by in vitro systems, dengue virus cross protection studies in sub-human primates will be performed. Observations will be made on dengue infection in monkeys.

Progress. Studies undertaken during the report period (July 1967-30 June 1968) were concerned with a) dengue virus cross protection studies in sub-human primates and b) laboratory observations during primary and heterologous dengue infections in rhesus monkeys.

1. General. Four monkey species have been studied: Macaca irus - Eight animals used. Two each infected with strains of dengue 1,2,3, and 4 described in the 1967 Annual Report. None of these animals circulated virus. Neutralization tests of pre-infection serum showed that all animals had high titered neutralizing antibody to dengue 1 and/or dengue 2 viruses. Cercopithicus aethiops - Four Animals used. Pre-infection serum from each animal was found to be free of dengue 1-4 neutralizing antibody at 1:10 dilution. One animal each was infected with dengue 1, 3 and two strains of dengue 2. Results of viremia testing are shown in table 1. Erythrocebus patas - Five animals used. Each animal was free of dengue 1-4 neutralizing antibody at a 1:10 dilution of serum before infection. One animal each was infected with dengue 1, two different dengue 2 strains, dengue 3 and dengue 4. Animals inoculated with dengue 1 and 2 viruses circulated virus at low titer for a short period (see table 1). Macaca mulatta. - One hundred twenty-nine animals have been used; 93 were inoculated with

various dengue virus strains in cross protection studies, one animal was inoculated with 17D strain of yellow fever virus and then with dengue 4 and 2 viruses in sequence; one animal was inoculated with dengue 1 and 2 viruses simultaneously; 4 were inoculated with inactivated dengue 4 virus material (see below); 7 inoculated with dengue viruses for antibody production, 6 inoculated with dengue 2 and sacrificed at 12 hour intervals, 7 inoculated with dengue 1 and sacrificed at 12 hour intervals, 4 sensitized for anaphylaxis with egg white and 6 used for LLC-MK2 cell control inoculations.

2. Dengue cross protection studies in rhesus monkeys.

a. Primary infections. Ninety-three animals were inoculated subcutaneously with a single dengue strain and then bled nearly every day from 1 through 10 and again on days 21 and 42. Infection of animals, the quantity of blood removed, its handling and storage was described in 1967 annual report. For viremia tests blood was anticoagulated with approximately 12.5 units of heparin/ml final concentration. Plasma obtained following centrifugation was stored for a variable period at -70°C. When tested it was diluted to 10^{-1}, 10^{-2} and 10^{-3} in 20% heat inactivated agamma calf serum. Portions of each dilution were inoculated into 3 replicate 2 oz. plaque bottles with LLC-MK 2 monolayers. Plaque assay was as described in the previous annual report.

Infectious doses varied in several experiments. Generally, high infectious dose inocula were employed, doses ranging from 10,000 to 500,000 PFU; low infectious doses ranged from 8-20 PFU. When low infectious doses were used to infect monkeys, the dose is indicated with tabular data.

The number of animals inoculated with various dengue strains, the infectious doses and those developing viremia on one or more days, is summarized in table 2. The infection experience of every animal studied is also provided in appendix i. Viremia followed primary dengue 1 and 2 infection in every animal; in 9 of 13 animals infected with dengue 3 virus and 26 of 29 animals infected with tissue culture passage dengue 4 (4328S). An additional 8 animals were infected with tissue culture passage dengue 4 but were bled only on alternate days from day 1-10 following infection. Viremia was detected on one or more days in 5 of these animals.

The daily incidence of detectable viremia following infection with dengue viruses types 1-4 is shown in table 3. Viremia with 1950-63 dengue 2 strain is not shown. The extremes of the period of viremia following infection are indicated by the boxes; the highest frequency of viremia in animals tested is indicated by a circle. Although the numbers are small, data suggest the onset of viremia to be later and the period of viremia to be shorter in animals infected with the low as compared with high infectious doses. The highest frequency of occurrence of viremia was day 5 in animals infected with high doses of dengue types 1-3 and day 4 with dengue type 4. Viremia titers varied between approximately 1×10^{-1} and 1×10^{-3}. Representative viremia responses were reported in the 1967 annual report.

b. Secondary dengue challenge.

The sequence, interval and results of challenging monkeys with heterologous or homologous dengue viruses is summarized in table 4.

The number of replicate experiments at each infection interval was very small. The following tentative conclusions are suggested: 1) All

animals resisted homologous challenge. This included 5 animals infected primarily with either 16681 or 1950-63 and challenged with the heterologous dengue 2 strain. Antibody response to homologous challenge differed in most instances from that in an animal with a viremic heterologous dengue infection (see below). 2) Dengue types 1, 2 and 4 cross protected against the employed dengue 3 strain at every infection interval (cross-protection by dengue 4 infection against dengue 3 challenge was not tested at 6 weeks). 3) Dengue 3 infection cross-protected against dengue 1 challenge at 6 weeks but not at 3 months. 4) Dengue 2 infection did not protect against dengue 1 challenge at 6 weeks, but did at 3 and 6 months. 5) Dengue 1 did not cross protect against dengue 2 at 6 weeks and 3 months, but did at 6 months. This was the only virus to cross protect against dengue 2 at any interval tested.

The duration and incidence of viremia in each monkey which circulated virus during secondary challenge are tabulated in table 5. Again, the number of replicate experiments is so small that interpretation of results is hazardous. It will be noted that animals secondarily challenged with dengue 1 three months after primary infection had a period of viremia considerably shorter than that during primary dengue 1 infections. Duration of secondary dengue 2 viremias was not different than those of primary infections when animals were infected at 6 week and 3 month intervals after primary infection; at 6 months, however, the frequency of detected viremia appeared lower than during primary infection. Each of these observations suggests occurrence of cross protection which increases with time against dengue type 2 by dengue 3 and 4 pre-infected animals and

against dengue type 1 by animals previously infected with dengue types 2, 3 and 4.

c. Tertiary dengue challenge. Table 6 summarizes the sequence of infections in monkeys infected with three different dengue viruses. Intervals between infections are shown in appendix 1. As shown, an animal infected with dengue 1 then dengue 3; and another animal infected with dengue 4 and then 3, each then challenged with dengue 2 circulated virus. These animals had low titered dengue 2 viremia, respectively, on days 2, 4 and 5 and days 3, 4 and 5 after infection. Neither had circulated dengue 3 during its secondary infection experience. Three other animals with the same sequence of virus infections did not circulate dengue 2 on tertiary challenge.

3. Antibody responses to primary infection and following challenge with homologous and heterologous viruses.

Circulating antibodies have been determined by HI and CF microtests, using a total volume of reagents of 4 and 6 drops, or 0.1 ml and 0.15 ml, respectively. However, due to the fact that the CF tests have not progressed as yet far enough, only the HI test results are reported at this time.

In order to remove non-specific inhibitors from the sera used in the HI tests, the kaolin treatment method was used throughout. This method was adopted generally in the investigation in preference to acetone treatment, as being more practical in handling large numbers of sera and as it eliminates slippage of the patterns that so often is noticed with the low dilutions of negative sera treated with acetone when tested with group B antigens. Furthermore, preliminary tests were done to ascertain the effec-

tiveness of the 2 methods, kaolin and acetone; all the serial sera from 3 animals and selected samples from 6 or 7 additional ones were treated in parallel with acetone and kaolin. No substantial or systematic difference in titers of antibodies was noticed between the 2 procedures.

All sera were tested in increasing 2-fold dilutions beginning at 1:10 against 8 units of the following antigens; dengue types 1,2,3 and 4, yellow fever, Zika, St. Louis, West Nile and Wesselsbron. In the few occasions when the final titrations showed either more or less (16 or 4 units) antigen present, the titers of the sera were corrected accordingly; all titers given in this report refer, therefore, to 8 units of antigen.

The monkeys used in the experiments had been pretested by HI and neutralization plaque test; they were devoid of circulating antibodies.

a. Primary response. Four monkeys infected with dengue 1, 6 with dengue 2,4 with dengue 3, and 6 with dengue 4 have thus far been studied. Samples of serum were taken almost daily during the first 10 days after infection, at wider intervals thereafter. For details concerning the strains used and viremia patterns following primary infections, see the 1967 Annual Progress Report.

Representative HI antibody responses to primary dengue 1,2,3 and 4 viruses are shown in tables 7-10. As of the 10th day after infection, no animal had detectable circulating antibodies except 2, which were positive for the first time on that day. One, following inoculation of dengue 1, reacted with a titer of 1:10 against Zika antigen; the other after infection of dengue 2, reacted at dilutions 1:20 with dengue 2 and 1:10 with Zika and SLE.

The next blood sample after the 10th day was taken on the 16th day from monkeys with dengues 1, 2 and 3; on the 12th day, from monkeys with dengue 4.

For the sake of brevity, the essential results of the HI tests have been summarized and are given in table 11. The table shows composite results; this procedure seemed justified in view of the fact that individual deviations from the mean values given were not out of line.

The main conclusions to derive from these results are: 1) Cross-reactive titers with non-dengue antigens, particularly SLE, Wesselsbron and Zika, are often as high as homologous titers in monkeys infected with dengues 1 and 2. 2) Within the dengue systems mean values as well as individual homologous titers were higher than heterologous titers. 3) During the period covered by the observations, from 0 to 50 days, post-challenge, the highest mean titers, whether homologous or heterologous, with any antigen were between 1:160 and 1:320 with monkeys infected with dengue 1; between 1:320 and 1:640 for dengue 2; between 1:40 and 1:80 for dengue 3 and 1:80 for dengue 4.

b. Secondary response. The number of combinations possible, when taking into account the succession of dengue types and 3 time intervals between challenges, 1 1/2, 3-4 and 6 months, is 48. At this time, HI tests have been completed with serum samples taken daily for 8 or 9 days after the secondary infection from monkeys challenged as shown here:

Primary	Interval, months	Secondary
dengue 1	4	dengue 2
"	4	dengue 3
dengue 2	4	dengue 1
"	4	dengue 3
dengue 3	4	dengue 1
"	4	dengue 2
"	1 1/2	dengue 3
dengue 4	1 1/2	dengue 2
"	4	dengue 4

As with the primary response, no attempt will be made to give here every detail of the experimental outcome. Instead, pertinent results are shown which illustrate certain discernible patterns of the secondary response in its earliest period, up to 8-9 days from challenge. These patterns are seen in tables 12-14:

1) No measurable response, or hardly any, to the second virus. This occurred when the first virus was dengue 2 and the second, dengue 1 or 3 (table 12).

2) Moderate secondary response. This took place when the first and second viruses were both dengue 3 or dengue 4 (table 13).

3) Marked secondary response. This was noted in cases with secondary viremia; when dengue 2 was the second virus, the first one being dengue 1, 3 or 4. This marked response also occurred with dengue 3 followed by dengue 1 (table 14).

It is still too early in the course of this work to derive general conclusions concerning the impact on the secondary response of the various combinations; it would seem, however, that some are more successful than others in bringing about an accelerated and marked antibody rise. It may well be, as suggested in viremia studies, that if dengue 2 or 1 is given first it prevents multiplication of dengues 3 or 4, given subsequently; as a result the secondary response is due to the mass of the antigen in the inoculum and consequently is weak. On the other hand, dengue 3 or 4 do not effectively prevent multiplication of dengue 2, so that the latter multiplying in a sensitized animal, brings about a sharp increase in the circulating antibodies.

The titers of circulating antibodies 8 days after the secondary infection, in the responsive combinations, were not excessively higher than those that resulted, at any time, after primary inoculation of either dengue 1 or 2. The striking feature was that in these responsive combinations signs of an immune response were seen in many animals by the 5th day after secondary challenge, and in all by the 8th day. This is in sharp contrast with the primary response in animals after primary dengue infection (see above); no immune response was detectable by the 10th day. Subsequent to the 8th day, antibody levels may increase to titers approximating those observed in dengue hemorrhagic fever patients in Thailand (i.e.≥ 1:10,240). This is illustrated in table 15. In this instance, antibody titers were highest against non-dengue antigens.

4. <u>Laboratory studies on venous blood following primary dengue infections and challenge with heterologous viruses.</u>

Test methods for white blood cell and platelet counts, hematocrit

determination, total protein, and prothrombin time have been described in the 1967 Annual Progress Report.

Total serum complement determinations were done by a quantitative method employing a Coleman spectrophotometer. When less than 10% or greater than 80% light was transmitted at 425λ (light transmission being inversely proportional to amount of hemolysis) fluctuations in complement level could not be measured accurately. Between these limits satisfactory assays could be made. In most instances, a 1:30 dilution of monkey serum produced 50% hemolysis (light transmission). Tests were performed as follows: To 0.5 ml of a 1:30 dilution of serum 1.5 ml veronal buffer and 1.0 ml sensitized sheep RBC were added. Tubes were incubated at 37°C for 30 min. They were then centrifuged for 10 min. at 1000 rpm and the supernatant read in the spectrophotometer. Values were expressed as optical density. Variations in amount of complement are directly proportional to variations in optical density.

a. White blood cell count. No attempt was made to establish normal values for monkeys or to define proportional changes from base line values which would be considered leucocytosis or leucopenia. The following terms are defined: leucopenia - sustained reduction of WBC from previous value occurring on 3 or more successive days; leucocytosis - sustained elevation of WBC above previous value occurring on 3 or more successive days.

Animals showing "leucopenia" during primary or secondary dengue infections are shown in table 16. It was notable that leucopenia occurred more frequently during primary dengue 2 and 4 infections than during dengue 1 or 3 infections. The difference between leucopenia in dengue 2 infections contrasted with dengue 1 and 3 infections was significant ($.05 > p > .01$). Leucopenia was noted during heterologous secondary dengue 2

infection in 24/48 animals but somewhat less frequently than in other sequences when dengue 2 infection followed dengue 4.

Leucocytosis occurred less frequently than did leucopenia responses in primary dengue infections (table 17). However, leucocytosis was noted during secondary dengue 1 and secondary dengue 2 infections at a somewhat higher frequency than during primary infections with these two viruses. Differences are not significant. Mild leucocytosis is a regular feature of secondary dengue hemorrhagic fever in Thailand.

b. Platelet count. No attempt was made to establish normal values. Thrombocytopenia was defined as a decrease in platelet count from pre-infection value occurring on 2 or more consecutive days during the 10 days following infection.

Incidence of "thrombocytopenia" during primary and secondary dengue infections is summarized in table 18. The frequency of thrombocytopenia during secondary dengue 2 infections was significantly higher than during primary dengue 2 infections ($.05>p>.01$).

c. Hemoconcentration. Hemoconcentration was defined as an elevation of hematocrit from previous value on 2 or more successive days followed by further decline in value. Hematocrit values generally declined over the period of bleeding, since 4 ml of blood was removed from the animal daily for eleven days. Among animals infected primarily and animals infected secondarily no significant difference in hemoconcentration was noted in any group or in any combination of virus infections.

d. Hypoproteinemia. Hypoproteinemia was defined as a decrease in total serum protein (measured by refractometer) from base line value

occurring on 2 or more successive days. Of animals infected primarily and animals infected secondarily no significant difference in incidence of hypoproteinemia was observed between groups or in any combination of infections. Considerable variation occurred in total proteins in serial samples from single animals. This variation appeared to be random and could not be explained by the condition of the specimen, the amount of hemolysis, etc. All specimens were temperature standardized. Distilled water readingswere made frequently. We consider the values obtained by refractometer to be unreliable. If a suitable quantitative micromethod can be found, all samples will be retested.

e. Prothrombin times. No prothrombin time tests were performed during the report period.

f. Total complement. Serial bleedings from 14 animals during first and second infections have been studied. Infection sequences included were as follows: d3-d1, two animals; d1-d3, two animals, d3-d3, one animal; d4-d2, eight animals and 17D yellow fever, d4-d2, one animal.

Although some unexplained fluctuation in complement levels occurred in serial samples from the same animal obtained over a period of up to 6 months, two patterns of complement response appeared to correlate with the period of acute experiment: 1) Increase in total complement. This reaction was seen in 4 animals bled on 10 successive days, then on days 21 and 42 after inoculation, after receiving subcutaneously a suspension of tissue culture cells prepared in the same manner as for dengue seed virus preparation. Response is illustrated in Figure 1. Elevation in total complement generally began in the third bleeding (2nd day after inoculation)

and declined to base line value by about the 8th or 9th bleeding. The magnitude of increase was of the order of 50%. An increase in complement was also noted in 12 out of 14 animals during primary dengue infections with dengue types 1-4 viruses. Increase in complement without a marked decrease was also seen in one animal receiving dengue 3 two times, in both animals infected with dengue 3 then dengue 1, and in both animals infected with dengue 1 then dengue 3. 2) Decrease or marked fluctuation in total complement. This response, illustrated in Figure 1, was observed in 6 of 8 monkeys during a dengue 2 infection which followed dengue 4 at an interval of between 6 weeks and 3 months. In all but one monkey a marked decrease in complement was detected first on day 3 after infection. In general, the longer the infection interval, the greater the depression of complement.

g. Observations on monkey 13177. Marked laboratory abnormalities were noted in one animal whose infection sequence is described in Figure 2. This animal, a 5 pound young adult female, was infected with 3000 PFU of dengue 4 virus. No virus was detected at a 1:10 dilution of plasma obtained on days 2, 4, 6, 8, 10 or 12 following infection. The animal had HI antibody to dengue 4 first detected at a 1:40 dilution in the 21 day post-infection specimen. Three months following primary dengue infection, 100,000 PFU of dengue 2 virus was inoculated subcutaneously. A few dengue 2 plaques were observed at a 1:10 dilution on day 2 of infection, no virus was detected on day 3, plaques were observed at all dilutions through 10^{-4} on days 4 and 5. No blood sample was obtained on day 6, but from day 7 through day 10 no further viremia was detected. On day 3 the monkey evolved, very suddenly, high titered heterologously reactive HI antibody

(response to dengue 2 is shown, titers with other antigens were similar). On days 4 and 5 this antibody was absent, possibly removed by combination with the virus produced. On day 7 a small amount of antibody was detectable and this rose rapidly during the next 48 hours. Laboratory abnormalities observed during infection were leucocytosis, thrombocytopenia, elevation of prothrombin time, decrease in complement, hypoproteinemia and an early unsustained elevation in hematocrit. It is notable that most laboratory abnormalities observed were initiated or became maximal during the period of antibody excess immediately following the termination of detectable viremia. In addition, a single thrombocytopenic spike and a single elevation of hematocrit to a value above base line was observed during the first period of antibody excess on day 3.

These abnormalities recapitulate most of the important laboratory findings observed in humans with the dengue shock syndrome (DSS). Laboratory values from a representative human case are shown in Figure 3. Note the onset of shock, thrombocytopenia, mild leucocytosis, hypoproteinemia and elevated prothrombin time on the 3rd day after onset of fever. It has been shown for humans that fever begins about 1 day after onset of viremia. The observed laboratory abnormalities in the monkey began 4 or 5 days after onset of viremia. This would be 3 or 4 days after onset of fever, or about the time that most DSS patients develop clinical and laboratory abnormalities. Thus, the sequence and timing of host response in monkey 13177 and man appear to be identical.

It is of interest that the abnormalities in host response observed in this and other monkeys during secondary dengue infections resemble in

kind the laboratory values described in rhesus monkeys during anaphylactic shock, particularly delayed anaphylactic shock, induced by egg albumin.

Whether these similarities imply common pathogenetic mechanisms remains to be determined. Attempts have been made in 30 additional animals to recapitulate all of the abnormalities observed in monkey 13177. These have been unsuccessful. Thus far, this animal has had the lowest antibody response to primary dengue 4 infection of any studied. Whether this or other factors are important in sensitizing animals to markedly abnormal host responses during secondary dengue infection remain to be investigated.

Summary and Conclusions.

1. Varying degrees of cross protection were induced by dengue infections in rhesus monkeys.

a. Dengue viruses types 1 (16007), 2 (16681), and 4 (4328S) completely protected monkeys against viremic response with a dengue type 3 virus (strain 16562) when inoculated subcutaneously at 6 weeks, 3-4 months and 6 months after primary infection.

b. Cross protection between two dengue viruses at only a short challenge interval was noted with dengue 3 followed by dengue 1. This was the only instance of the type of early cross protection described by Sabin in human volunteers infected with dengue 1 or 2 viruses and challenged with the heterologous virus type.

c. Cross protection increasing with time was noted between dengue types 2, 3 or 4 to subsequent challenge by dengue type 1 or

or between dengue 1, 3 and 4 to subsequent challenge by dengue 2. Dengue type 1 infection completely protected 2 monkeys against a viremic response with dengue type 2 virus at a 6 month interval.

d. Of all viruses tested, dengue type 2 was least protected. This observation is consistent with the data from Bangkok studies which showed that dengue type 2 viruses were more frequently isolated from symptomatic secondary dengue infections than other viruses. If infection with dengues other than type 2 are more frequently prevented by previous heterologous virus experience, type 2 infections would be expected to predominate in observed secondary dengue illnesses.

e. When monkeys were infected with two different dengue viruses, then challenged a third time, in all but two instances viremia was completely prevented. In these two instances dengue 3 was the second virus challenge. These data suggest that sequential vaccination by attenuated dengue 1 then dengue 2 will protect against homologous challenge as well as dengue 3 and 4 infection (complete dengue protection). This might be a relatively simple vaccination procedure particularly applicable to military populations. Before applying these data to man further demonstration is required in monkeys that 1) protection from two dengue infections to any subsequent challenge is of long duration and 2) that attenuated dengue 1 and 2 virus vaccine strains suitable for human use will produce the same degree of cross protection as has been demonstrated above.

2. In general, antibody responses in monkeys and man appear to be very similar. When viremia occurred during a secondary dengue infection, a marked secondary-type antibody response was noted. When a primary dengue infection protected an animal against viremic infection with a

second virus, antibody response was minimal. Intermediate "booster responses" were noted when animals were re-inoculated with homologous virus. It is probable in the latter two instances that the antigenic stimulus was derived from the virus inoculum only. Apparently, when antigens were identical the booster effect is greater than if they are only closely similar. In future studies antibody responses might be used instead of virus isolation procedures to test the efficacy of cross protection against dengue strains.

3. Various laboratory abnormalities were noted during primary and secondary dengue infections.

a. A high percentage of monkeys infected with dengue 2 viruses had a period of leucopenia coinciding, in most instances, with viremia.

b. Mild thrombocytopenia occurred significantly more frequently in secondary dengue 2 infections than in primary dengue 1-4 infections.

c. Increase in serum complement of short duration was noted in serial bleedings from monkeys injected with control tissue culture extract or primarily infected with dengue 1-4 viruses. Increase in serum complement also occurred in two experimental periods in one monkey given dengue 3 virus twice, two monkeys infected with dengue 3 then dengue 1 and two monkeys given dengue 1 then dengue 3.

d. A sharp decrease in serum complement was observed on or about day 3 after a secondary dengue 2 infection in 6/8 monkeys previously infected with dengue 4. This observation plus that reported in (b) establishes that at least two blood or serum laboratory values differ during secondary dengue infections as compared with primary infections.

e. A single animal developed hypoproteinemia and elevation in prothrombin time in addition to thrombocytopenia, mild leucocytosis and decreased complement during a secondary dengue 2 infection. The sequence of abnormalities in this animal recapitulated in time and in kind those observed in human dengue hemorrhagic fever.

It may not be unreasonable to hypothesize that decrease in serum complement, thrombocytopenia and mild leucocytosis observed in monkeys may be related to antigen-antibody complexing, constituting a mild form of dengue hypersensitivity disease.

Table 1. Tests for viremia in grivet and patas monkeys infected subcutaneously with dengue viruses.

Species	Animal number	Dengue type	Strain	Inoculum PFU	Viremia on indicated day after infection[1] 1	2	3	4	5	6	7	8	9
Grivet	13099	d1	16007	10^5		0	0	0	0	+	+	ND	0
	13101	d2	1950-63	10^5		0	0	0	0	0	+	ND	0
	13103	d2	16681	10^6		0	0	+	+	+	0	ND	0
	13102	d3	16562	$10^{4.5}$		0	+	0	0	0	0	ND	0
Patas	12641	d1	16607	10^5		0	0	0	0	0	0	+	0
	12666	d2	16681	10^6		0	0	0	0	+	0	0	0
	12669	d2	1950-63	10^5		0	0	0	0	0	+	0	0
	12670	d3	16562	$10^{4.5}$		0	0	0	0	0	0	0	0
	12671	d4	4328 S	$10^{3.3}$		0	0	0	0	0	0	0	0

[1] 0 = no plaques observed in 3 two ounce bottles inoculated with 0.5 ml of 10^{-1} dilution of plasma.

\+ = one or more plaques observed under above described conditions.

ND = blood not collected.

Table 2. Incidence of viremia (days 1-10 after infection) in rhesus monkeys during primary dengue virus infections.

Dengue type	d1	d2		d3	d4		
Strain	16007	16681	1950-63	16562	4328 S	4328 S	4391
Host passage	TC psg	TC psg	TC psg	TC psg	TC psg	sm psg	sm psg
Dose (PFU) >10,000	11/11*	13/13	4/4	7/11	26/29	3/5	0/4
Dose (PFU) <50	2/2	2/2	2/2	2/2			
Total	13/13	15/15	6/6	9/13	26/29**	3/5	0/4

* animals with viremia/no. tested

** Eight animals bled only on alternate days not tabulated.

Table 3. Daily frequency of viremia during primary dengue infections in rhesus monkeys.

Virus	approx. PFU	Day after infection 1	2	3	4	5	6	7	8	9	10
16007 (d1)	>10,000	1/7*	3/11	7/11	7/11	5/5	8/9	3/4	6/9	1/9	0/10
	<50		0/2	0/3	1/2	1/2	2/2	2/2		2/2	2/2
16681 (d2)	>10,000	0/7	6/11	9/11	8/11	4/4	7/9	3/4	1/9	0/9	0/10
	<50		0/2	1/2	1/2	1/2	2/2	2/2		0/2	0/2
16562 (d3)	>10,000	0/5	2/7	2/7	2/6	3/6	0/5	2/5	0/5	0/5	0/6
	<50		0/2	0/2	1/2	1/2	2/2	2/2		0/2	0/2
4328 S (d4)	>10,000	5/25	13/26	14/26	19/25	10/21	6/19	9/25	4/25	1/25	0/25

* no. with detectable virus/ no. specimens tested.

Table 4. Incidence of viremia during secondary dengue infections in rhesus monkeys.

Primary infecting virus		6 week interval between primary and secondary infection			
		Secondary infecting virus			
		d1	d2	d3	
d1		(0/1)*	2/2	(0/2)	
d2		2/2	(0/1)	(0/2)	
d3		(0/2)	2/2	(0/1)	
d4			3/3		
		3-4 month interval between 1° and 2°			
		d1	d2	d3	
d1			2/2	(0/3)	
d2		(1/3)	(0/2)	(0/3)	
d3		2/2	2/2		
d4		2/2	26/26	(0/2)	
		6 month interval between 1° and 2°			
		d1	d2	d3	d4
d1		(0/1)	(0/2)	(0/1)	
d3		(0/3)		(0/2)	(0/2)
d3			4/4		
d4		(1/2)	3/3	(0/2)	
	Viremic animals / Total heterologous challenges	8/16	44/46	0/17	0/2

* animals with viremia/ total tested. Circled fractions indicate protection or partial protection.

Table 5. Viremia during days 1-10 in secondary dengue infections in rhesus monkeys.

Interval	Infection sequence	Day after infection 1	2	3	4	5	6	7	8	9	10
6 weeks	d1-d2	0/2	0/2	1/2		2/2*	2/2	0/2	0/2	0/2	0/2
	d2-d1	0/2	0/2	1/2	0/1	1/2	1/2	1/2	2/2	0/2	0/2
	d3-d2	0/2	0/2	0/2		2/2	2/2	0/2	0/2	0/2	0/2
	d4-d2	1/4	1/4	1/4	4/4	3/3	4/4	1/4	0/4	0/2	0/2
3-4 months	d1-d2	0/2	1/2	1/2	1/2	2/2	1/2	0/2	0/2	0/2	0/2
	d2-d1	0/1	0/1	1/1		0/1	0/1	0/1	0/1	0/1	0/1
	d3-d1	0/2	0/2	0/2		2/2	1/2	0/2	0/2	0/2	0/2
	d3-d2	0/2	1/2	1/2	1/2	2/2	1/2	0/2	0/2		0/2
	d4-d1	0/2	0/2	1/2	2/2	2/2	1/2	0/2	0/2	0/2	0/2
	d4-d2	1/17	8/18	11/18	15/18	13/17	7/17	4/18	1/18	1/18	0/18
6 months	d3-d2	0/4	1/4	2/4	4/4	2/2	1/4	0/4	0/4	0/4	0/4
	d4-d2	0/2	0/2	0/2	2/2	1/2	1/2	1/2	0/2	0/2	0/2
Primary infect.	d1 only	1/7	3/11	7/11	7/11	5/5	8/9	3/4	6/9	1/9	0/10
	d2 only	0/7	6/11	9/11	9/11	4/4	7/9	3/4	1/9	0/9	0/10

* No. with detectable virus/no. specimens tested. Circled fractions indicate day after infection of highest frequency of viremia. Squares indicate limits of period of viremia in monkeys infected with same virus.

Table 6. Incidence of viremia during tertiary dengue infection of rhesus monkeys.

Primary-secondary sequence	Third challenge virus				
	d1	d2	d3	d4	
d1-d2			0/2	0/4	
d1-d3		1/3		0/2	
d2-d1			0/2	0/3	
d2-d3	0/1			0/1	
d2-d4	0/1		0/1		
d3-d1		0/2		0/1	
d3-d2	0/3			0/5	
d4-d1		0/2	0/2		
d4-d2	0/5		0/4		
d4-d3	0/1	1/2			
	0/11	2/9	0/11	0/15	Total 2/46

Table 7. Hemagglutination-inhibition test. Response in monkey #13015 following primary infection with dengue 1 (16007).

Serum	Antigen, 8 units							
day after infection	D 1	D 2	D 3	D 4	SLE	WN	ZIKA	YF
PRE	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
16	80	10	20	10	10	10	40	20
30	160	40	80	40	20	20	160	40
52	160	20	40	20	20	20	80	20

Reciprocal of serum titer; 0, no inhibition at dilution 1:10, lowest used.

Table 8. Hemagglutination-inhibition test. Response in monkey #13017 following primary infection with dengue 2 (1950-63).

Serum day after infection	Antigen, 8 units							
	D-1	D 2	D 3	D 4	SLE	WN	ZIKA	YF
PRE	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0
10	0	20	0	0	10	0	10	0
16	20	160	40	40	160	20	160	20
30	40	320	40	80	160	40	320	20
52	40	320	40	40	160	40	320	40

See Table 7

Table 9. Hemagglutination-inhibition test. Response in monkey #13018 following primary infection with dengue 3 (16562).

Serum	Antigen, 8 units							
day after infection	D 1	D 2	D 3	D 4	SLE	WN	ZIKA	YF
PRE	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0
5	0	0	0	0	0	10	0	0
6	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0
16	0	10	80	10	40	10	40	10
30	0	10	40	10	40	10	40	0
52	10	10	80	10	40	10	40	10

See Table 7.

Table 10. Hemagglutination-inhibition test. Response in monkey #13179 following primary infection with dengue 4 (4328 S).

Serum	Antigen, 8 units							
days after infection	D 1	D 2	D 3	D 4	SLE	WN	ZIKA	YF
PRE	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0
12	0	0	0	10	0	0	0	0
21	20	40	40	320	80	40	160	80
42	10	20	20	160	40	40	160	20
122	0	10	10	80	40	20	80	10

See Table 7.

Table 11. Primary antibody response by hemagglutination-inhibition in monkeys subcutaneously inoculated with dengue viruses. Combined results and mean titer values

Virus	Days After Inoculation	Antigen, 8 units								
		D 1	D 2	D 3	D 4	YF	ZIKA	SLE	WN	WESS
Dengue 1	16	*5	3	3.5	3.5	3	5	1	-	4
	19	5.5	3.5	3.5	5	3	6	4	5	5
4 monkeys	30	5	3.5	3.5	4	2.5	5	3	-	4
	41	5	3	3.5	4	2.5	5	3.5	5	5
	52	5	3	3.5	3	2	4	2	-	3
Dengue 2	16	1	5	2.5	3	1.5	4	4	-	3.5
	19	3.8	7	4.3	6.8	4.3	6.5	6.3	6.0	6.5
6 monkeys	30	1.5	5	2	3	1	4	4	-	3.5
	41	4.5	6.8	4	6.3	4	6	6	5	6.5
	52	2	6.5	3	3	2	5	4.5	-	4.5
Dengue 3	16	0	0.5	3.5	1	0.5	1.5	2	0.5	2
	19	0	0	2	0	0	0	0.5	0	0
4 monkeys	30	0.5	0.5	3	1	1	2	2	0.5	2
	41	0	0	2	0	0	1	1	0	0.5
	52	0.5	0.5	3	1	0.5	2	2	0.5	2
Dengue 4	12	0	0	0	0.8	0	0.3	0.3	0.3	0.3
6 monkeys	21	1.5	2	1.7	5	2	3	2	1	3.3
	42	0.7	1.2	1	3.9	1.2	3.2	3.2	2	2.9

* For each day an antigen is given the mean titer of the sera tested. The titers are expressed as: 1 for 1:10, 2 for 1:20, 3 for 1:40, 4 for 1:80, etc.

Table 12. Infection with dengue viruses. Hemagglutination-inhibition. Challenge by second virus resulting in no or hardly detectable response within 8 days.

Antigen	Sequence of dengue viruses and days after second inoculation											
8 units	Type 2 followed by 3 Monkey #13024				Type 2 followed by 1 Monkey #13028				Type 1 followed by 3 Monkey #13014			
	0	5	8	*change	0	5	8	change	0	5	7	change
Dengue 1	#3	3	3	0	5	5	6	1	6	6	6	0
Dengue 2	7	7	7	0	7	7	7	0	4	4	5	1
Dengue 3	4	4	4	0	4	4	4	0	4	4	5	1
Dengue 4	7	7	7	0	5	5	5	0	4	4	4	0
Yellow Fever	2	2	3	1	4	5	5	1	3	4	4	1
Zika	6	6	6	0	7	7	7	0	5	5	5	0
SLE	5	5	6	1	7	7	7	0	3	4	4	1
WN	8	8	8	0	4	5	5	1	2	2	3	1
Wesselsbron	6	6	6	0	7	7	7	0	5	5	5	0

* Change: increase in titer between 0 and 8th day expressed as number of 2-fold dilutions.

For titers, see Table 11.

Table 13. Infection with dengue viruses. Hemagglutination-inhibition Challenge by second virus resulting in a moderate response within 8 days.

Antigen 8 units	Sequence of dengue viruses and days after second inoculation							
	Type 3 followed by 3 Monkey #13215				Type 4 followed by 4 Monkey #13178			
	0	5	8	change	0	5	8	change
Dengue 1	0	0	1	1	1	1	2	1
Dengue 2	0	0	1	1	1	2	2	1
Dengue 3	2	4	4	2	2	2	3	1
Dengue 4	0	0	1	1	4	5	6	2
Yellow Fever	0	1	1	1	2	2	3	1
ZIKA	0	2	2	2	3	5	5	2
SLE	1	2	3	2	3	4	4	1
WN	0	1	2	2	3	4	4	1
Wesselsbron	1	2	3	2	3	4	4	1

See Table 12.

Table 14. Infection with dengue viruses. Hemagglutination-inhibition Challenge by second virus resulting in marked response within 8 days.

Antigen 8 units	Sequence of dengue viruses and days after second inoculation															
	Type 1, then 2* Monkey #13022				Type 3, then 1* Monkey #13019				Type 3, then 2* Monkey #13026				Type 4, then 2* Monkey #13172			
	0	5	8	change	0	5	7	change	0	5	8	change	0	5	8	change
D 1	6	6	8	2	0	0	2	2	0	0	1	1	0	0	3	3
D 2	4	4	8	4	0	0	2	2	0	0	2	2	0	0	3	3
D 3	4	4	6	2	1	2	4	3	2	2	3	1	0	0	3	3
D 4	4	4	6	2	0	0	3	3	0	0	1	1	3	3	4	1
YF	3	3	5	2	0	0	2	2	0	0	2	2	0	0	2	2
Zika	6	6	8	2	0	2	4	4	0	1	4	4	2	3	5	3
SLE	3	3	5	2	1	3	5	4	0	2	4	4	2	2	5	3
WN	5	5	7	2	0	0	2	2	0	0	1	1	0	0	3	3
Wess	5	5	7	2	0	2	5	5	0	1	3	3	1	2	5	4

See Table 12.

* Viremia detected during secondary infection.

Table 15. Antibody response by HI in rhesus monkey #13179 infected in succession with dengue 4 and dengue 2 at an interval of 112 days.

Days after		Antigen							
Primary	Secondary	d1	d2	d3	d4	SLE	ZIKA	YF	WESS
42		10*	20	20	160	40	160	20	80
119	7	40	80	80	160	160	320	80	320
121	9	320	640	640	640	1280	2560	640	2560
124	12	1280	2560	2560	2560	10240	10240	2560	10240

* reciprocal of serum titer.

Table 16. Frequency of leucopenia in rhesus monkeys during primary and secondary dengue infections.*

Virus Type	Secondary Infection: Primary Infecting Virus Type d1	d2	d3	d4	Primary Infection	Primary Infecting Virus Type
	%	%	%	%	%	
d1	0/2 (0)	2/7 (28.6)	3/4 (75)	0/4 (0)	4/12 (33.3)	d1
d2	4/6 (66.7)	3/5 (60)	5/8 (62.5)	15/34 (44.1)	13/19 (68.4)	d2
d3	2/5 (40)	2/6 (33.3)	0/1 (0)	0/4 (0)	3/12 (25)	d3
d4	0/0	0/1 (0)	0/0	1/2 (50)	21/46 (45.7)	d4

* See text for definition of leucopenia.

Table 17. Frequency of leucocytosis during primary and secondary dengue infections in rhesus monkeys.*

Virus type	Secondary Infection: Primary Infecting Virus Type d1		d2		d3		d4		Primary Infection		Primary Infecting Virus Type
		%		%		%		%		%	
d1	0/2	(0)	2/7	(28.6)	4/4	(100)	1/4	(25)	3/12	(25)	d1
d2	3/6	(50)	1/5	(20)	5/8	(62.5)	7/34	(20.5)	2/19	(10.5)	d2
d3	2/5	(40)	3/6	(50)	0/1	(0)	0/4	(0)	3/12	(25)	d3
d4	0/0		0/1	(0)	0/0		0/2	(0)	13/46	(28.3)	d4

* See text for definition of leucocytosis.

Table 18. Frequency of thrombocytopenia during primary and secondary dengue infections in rhesus monkeys.*

Secondary Infecting Virus Type	Secondary Infection: Primary Infecting Virus Type d1	d2	d3	d4	Primary Infection	Primary Infecting Virus Type
	%	%	%	%	%	
d1	1/2 (50.0)	1/7 (14.3)	2/4 (50.0)	2/4 (50.0)	3/12 (25.0)	d1
d2	4/6 (66.7)	1/5 (20.0)	4/8 (50.0)	17/34 (50.0)	4/19 (21.1)	d2
d3	1/5 (20.0)	0/6 (0.0)	1/1 (100.0)	3/4 (75.0)	4/12 (33.3)	d3
d4	0/0	0/1 (0.0)	0/0	0/2 (0.0)	11/46 (24.4)	d4

* See text for definition of thrombocytopenia.

Appendix 1. Dengue Viremia Studies in Rhesus Monkeys.*

Monkey #	1° Inoc. Virus	Interval	2° Challenge Virus	Interval	3° Challenge Virus	Interval	4° Challenge Virus
13014	1 (+)	3 1/2 mo	3 (0)	1 yr	4 (0)	3 1/2 mo	2 ( )
13015	1 (+)	3 1/2 mo	3 (0)	1 yr	2 (+)	3 1/2 mo	4 ( )
13016	2 (+)						
13017	2 (+)	3 1/2 mo	1 (+)				
13018	3 (+)	3 1/2 mo	1 (+)				
13019	3 (+)	3 1/2 mo	1 (+)	1 yr	2 (0)	3 1/2 mo	4 ( )
13020	2 (+)	3 1/2 mo	[2 (0)]	1 yr	[2 (0)]	3 1/2 mo	3 ( )
13021	2 (+)	3 1/2 mo	[2 (0)]	1 yr	[1 (0)]	3 1/2 mo	3 ( )
13022	1 (+)	3 1/2 mo	2 (+)				
13023	1 (+)	3 1/2 mo	2 (+)	1 yr	4 (0)	3 mo	3 ( )
13024	2 (+)	3 1/2 mo	3 (0)				
13025	2 (+)	3 1/2 mo	3 (0)				
13026	3 (+)	3 1/2 mo	2 (+)	1 yr	4 (0)		
13027	3 (+)	3 1/2 mo	2 (+)	1 yr	1 (0)	3 mo	4 ( )
13028	2 (+)	3 1/2 mo	1 (0)				
13029	2 (+)	3 1/2 mo	1 (0)				
13124	1 (+)	6 mo	2 (0)	3 mo	3 (0)	3 mo	4 (0)
13125	1 (+)	6 mo	2 (0)	3 mo	4 (0)	3 mo	3 (0)
13126	2 (+)	6 mo	1 (0)	3 mo	3 (0)	3 mo	4 (0)
13127	2 (+)						
13128	3 (+)	6 mo	2 (+)	3 mo	1 (0)	3 mo	4 (0)
13129	3 (+)	6 mo	2 (+)	3 mo	4 (0)		
13130	2 (+)	6 mo	1 (0)	3 mo	4 (0)	3 mo	3 (0)
13131	2 (+)	6 mo	3 (0)	3 mo	1 (0)	3 mo	4 (0)
13172	4 (+)	6 wks	2 (+)	1 yr	1 (0)	3 mo	3 ( )
13173	4 (0)	4 1/2 mo	2 (+)	3 mo	1 (0)	9 1/2 mo	3 ( )
13175	4 (+)	6 wks	2 (+)				
13176	4 (0)						
13177	4 (0)	3 mo	2 (+)				
13178	4 (+)	3 mo	[4 (0)]				
13179	4 (+)	3 1/2 mo	2 (+)	3 mo	3 (0)	10 mo	1 ( )
13180	4 (+)	3 1/2 mo	2 (+)	6 mo	1 (0)	10 mo	3 ( )
13181	1 (+)	6 1/2 mo	[1 (0)]				
13182	1 (+)	6 1/2 mo	3 (0)	3 1/2 mo	2 (0)	6 mo	4 ( )
13183	2 (+)	6 1/2 mo	[2 (0)]				
13184	2 (+)	6 1/2 mo	[2 (0)]	6 1/2 mo	[4 (0)]		1 ( )
13185	3 (+)	6 1/2 mo	2 (+)	3 1/2 mo	4 (0)	6 mo	1 ( )
13186	3 (0)	6 1/2 mo	2 (+)	3 1/2 mo	4 (0)	6 mo	1 ( )
13187	2 (+)	6 1/2 mo	1 (0)		4 (0)		3 ( )
13188	2 (+)	6 1/2 mo	3 (0)				
13190	4 (+)	2 mo	2 (+)				
13191	4 (+)	2 mo	2 (+)				
13192	4 (+)	3 mo	2 (+)				
13193	4 (+)	3 mo	2 (+)				
13201	1 (+)	6 wks	[1 (0)]				
13202	1 (+)	6 wks	2 (+)	6 mo	3 (0)	6 mo	4 ( )
13203	1 (+)	6 wks	2 (+)	6 mo	4 (0)	6 mo	3 ( )
13204	1 (+)	6 wks	3 (0)	6 mo	2 (0)	6 mo	4 ( )
13205	1 (+)	6 wks	3 (0)	6 mo	4 (0)	6 mo	2 ( )

Appendix i. Cont.

13206	2	(+)	6 wks	1	(+)	6 mo	3	(0)	6 mo	4 ( )
13207	2	(+)	6 wks	1	(+)	6 mo	4	(0)	6 mo	3 ( )
13208	2	(+)	6 wks	[2	(0)]	6 mo	[4	(0)]	6 mo	3 ( )
13209	2	(+)	6 wks	3	(0)	6 mo	2	(0)	5 mo	1 ( )
13210	2	(+)	6 wks	3	(0)	6 mo	4	(0)	5 mo	1 ( )
13211	3	(+)	6 wks	1	(0)	6 mo	2	(0)	5 mo	4 ( )
13212	3	(0)	6 wks	1	(0)	6 mo	4	(0)	5 mo	2 ( )
13213	3	(0)	6 wks	2	(+)	6 mo	1	(0)	5 mo	4 ( )
13214	3	(+)	6 wks	2	(+)	6 mo	4	(0)	5 mo	1 ( )
13215	3	(0)	6 wks	[3	(0)]		1	(0)		
13216	2/1	(+)								
13217	YF	(0)	6 wks	4	(+)	3 mo	2	(+)		
13239	4	(+)	3 mo	2	(+)	7 mo	3	(0)	3 mo	1 ( )
13240	4	(+)	3 mo	2	(+)	7 mo	3	(0)		
13241	4	(+)	3 mo	2	(+)	7 mo	4	(0)		
13242	4	(+)	3 mo	2	(+)	7 mo	1	(0)		
13243	4	(0)	3 mo	2	(+)	7 mo	3	(0)	3 mo	1 ( )
13244	4	(+)	3 mo	1	(+)	6 mo	2	(0)	3 mo	3 ( )
13245	4	(0)	3 mo	1	(+)	6 mo	3	(0)	3 mo	2 ( )
13246	4	(+)	3 mo	3	(0)	6 mo	1	(0)	3 mo	2 ( )
13247	4	(+)	3 mo	3	(0)	6 mo	2	(+)	3 mo	1 ( )
13248	4	(+)								
13249	4	(+)	6 mo	1	(0)	2 mo	3	(0)	3 mo	2 ( )
13250	4	(+)	6 mo	1	(+)	2 mo	2	(0)	3 mo	3 ( )
13251	4	(+)								
13252	4	(+)	6 mo	2	(+)	2 mo	1	(0)	3 mo	3 ( )
13253	4	(+)	6 mo	3	(0)	2 mo	2	(0)	3 mo	1 ( )
13254	4	(+)	6 mo	3	(0)	2 mo	1	(0)	2 mo	2 ( )
13255	4	(+)	6 mo	[4	(0)]					
13256	4	(+)	4 mo	2	(+)					
13257	4	(+)	4 mo	2	(+)					
13258	4	(0)	4 mo	2	(+)					
13289	4	(+)	3 mo	2	(+)					
13290	4	(+)	3 mo	2	(+)					
13291	4	(+)	3 mo	2	(+)					
13292	4	(+)	5 mo	2	(+)					
13293	4	(+)	5 mo	2	(+)					
13294	4	(+)	4 mo	2	(+)					
13295	4	(0)	4 mo	2	(+)					
13296	4	(0)	4 mo	2	(+)					
13297	4	(+)	4 1/2 mo	2	(+)					
13298	4	(+)	4 1/2 mo	2	(+)					
13299	4	(0)	3 mo	2	(+)					
13300	4	(0)	3 mo	2	(+)					
13301	4	(0)	3 mo	2	(+)					
13302	4	(0)	3 mo	2	(+)					
13305	BPL 4	(0)	3 wks	2	(+)					
13306	BPL 4	(0)	5 wks	4	(+)		2	( )		
13307	BPL 4	(0)	3 wks	2	(+)					
13308	BPL 4	(0)	5 wks	4	(+)		2	( )		

* - Figure in parenthesis indicates presence or absence of viremia during days 1-10 post-infection

\- horizontal line indicates monkey removed from study

\- enclosed figures are exceptions to legend at column head.

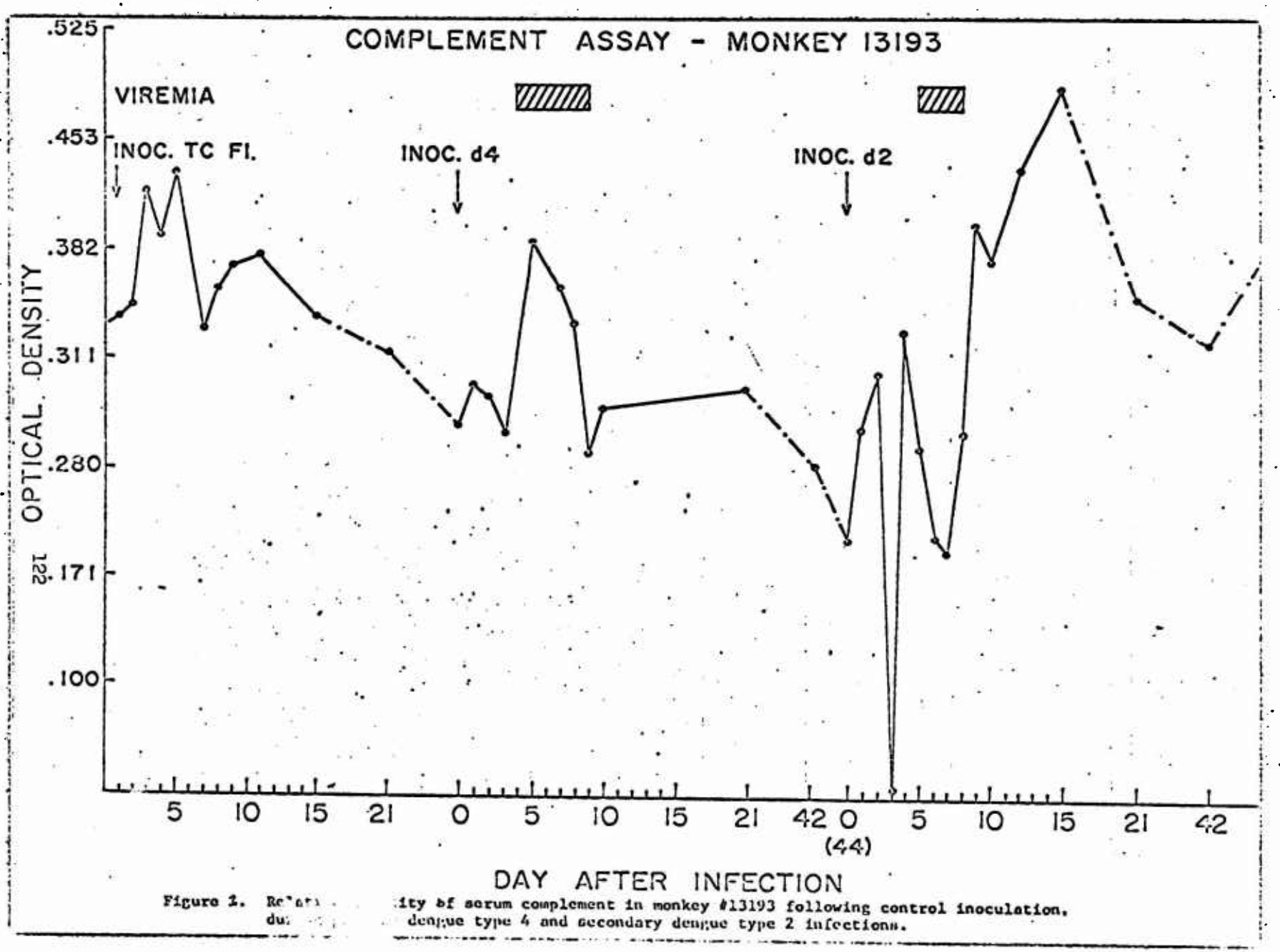

Figure 1. Re[illegible] ity of serum complement in monkey #13193 following control inoculation, du[illegible] dengue type 4 and secondary dengue type 2 infections.

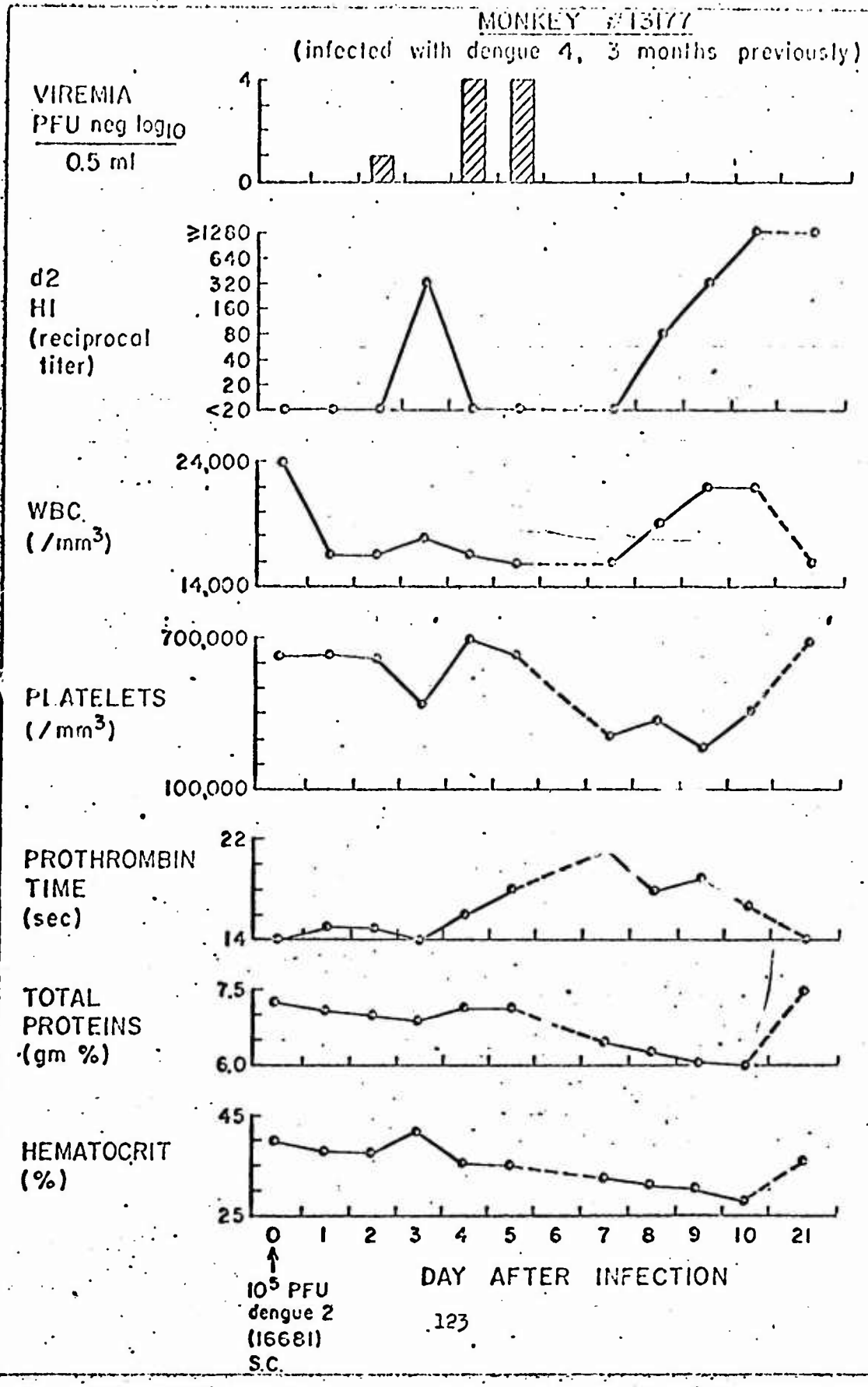

Figure 2. Laboratory responses during secondary dengue infection in a rhesus monkey infected with type 4 then type 2 viruses at an interval of 3 months.

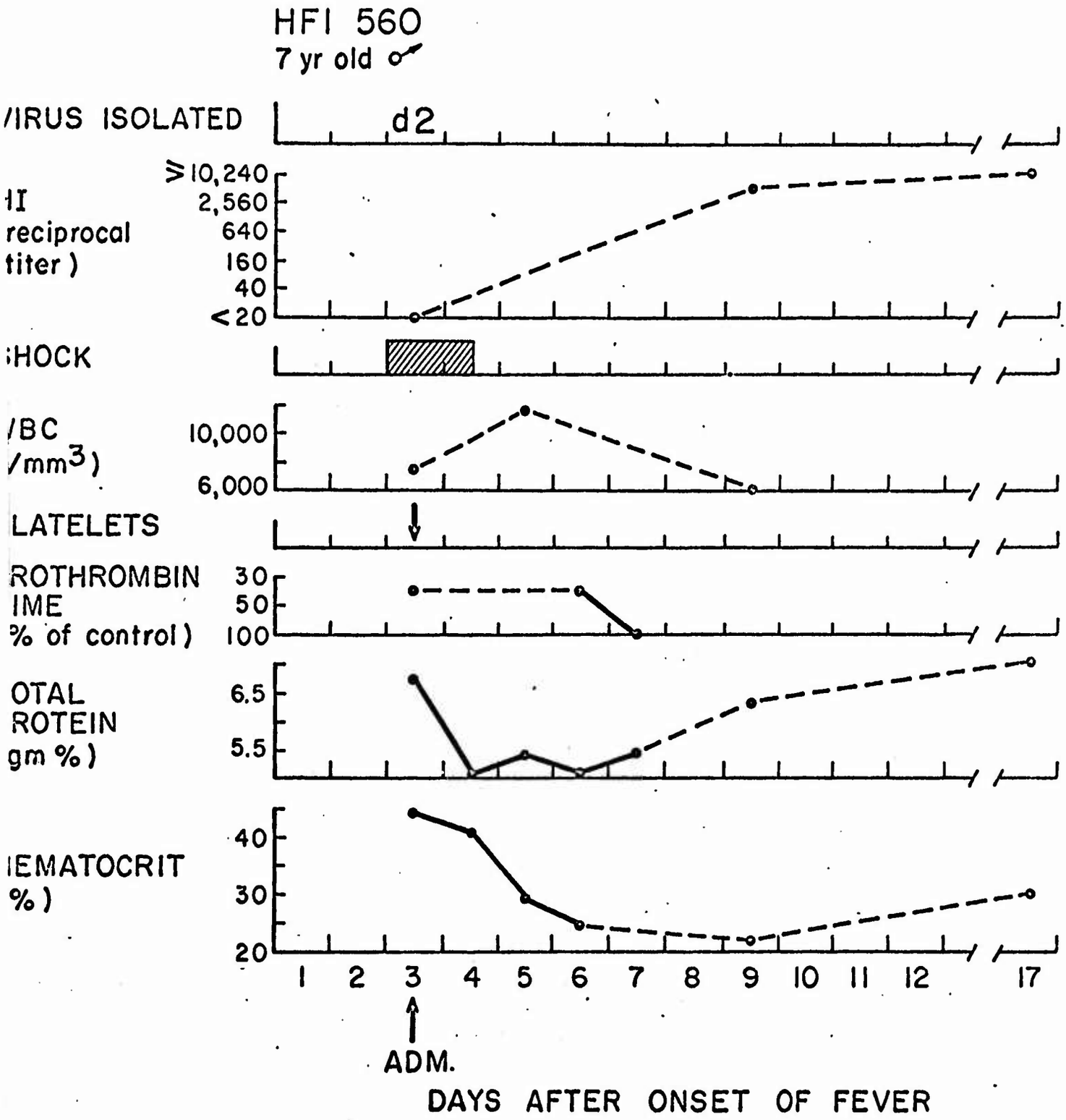

Figure 3. Laboratory studies on a patient hospitalized with dengue hemorrhagic fever in Thailand, 1964.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6SC9	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY (a. RDT, b. R&D)	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 6113001L 3A013001A91C 00 123

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) POPULATION GENETICS OF HEMOGLOBIN E, THALASSEMIA AND RELATED GENETIC POLYMORPHYISMS IN THAILAND TH23

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
010100 MICROBIOLOGY	11 65	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	c. PROFESSIONAL MAN-YEARS	d. FUNDS (In thousands)
	11 65			
	a. NUMBER DA 49 193 MD 2847			
B. CONTRACT	c. TYPE J.C d. AMOUNT $109,367	PRIOR FY 68	1	16
		CURRENT FY 69	1	8

19. GOVT. LAB/INSTALLATION/ACTIVITY

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20. PERFORMING ORGANIZATION

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21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NRAIR

23. KEYWORDS: POPULATION, GENETICS, HEMAGLOBIN.

24.

(U) TECH OBJECTIVE - TO PERFORM DETAILED EPIDEMIOLOGIC STUDIES IN THAILAND OF CERTAIN GENETIC TRAITS (INCLUDING VARIOUS HEMOGLOBINS AND BLOOD ENZYMES) AS THEY RELATE TO SUSCEPTIBILITY OR RESISTANCE TO SELECTED EPIDEMIC DISEASES.

(U) APPROACH- EXAMINATION OF SPECIMENS COLLECTED FROM FAMILY AND TRIBAL GROUPS IN THAILAND. SEROLOGIC AND BIOCHEMICAL STUDIES TO BE PERFORMED MAINLY IN THE UNITED STATES.

25. (U) PROGRESS - JUL 67 THRU JUN 68 ALL PROPOSED STUDIES IN 3300 THAIS HAVE BEEN COMPLETED. THE GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF THAILAND IS OF THE B ELECTROPHORETIC VARIETY AND THE DEFICIENCY IS OF THE SEVERE TYPE SEEN IN CAUCASIANS. THE HIGHEST DEFICIENCY RATE (14 PER CENT) IS IN CENTRAL THAILAND. OTHER GENE FREQUENCIES ARE NOW BEING STUDIED AND EFFORTS BEING MADE TO FIND CORRELATIONS. PRELIMINARY EVALUATION SUGGESTS MIGRATION PATTERNS MAY BE OF MUCH MORE IMPORTANCE IN EXPLAINING VARIATIONS THAN SELECTION DUE TO EXPERIENCE WITH DISEASES. FOR TECHNICAL REPORTS SEE ANNUAL REPORT TO USAMRDC ON CONTRACT NO. DA 49 193 MD 2847.

26.

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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 123, Population genetics of hemoglobin E, thalassemia, and related genetic polymorphisms in Thailand.

Investigators.
Principal: Donald L. Rucknagel, M.D.
Associate: Henry Gershowitz, Ph.D.; Supa Na-Nakorn, M.D.; Prawase Wasi, M.D., Ph.D.

Description.

The objectives of this project are (a) to establish in greater detail the frequency of various genetic hemoglobinopathies in Thailand; (b) to correlate the frequency of these with the prevalence of malaria, past and present; and (c) to assess their impact on other aspects of the health of the individual.

Progress.

(The following has been taken from a manuscript that was prepared for publication by the Principal Investigator and his associates).

Venous blood samples were drawn from over 3000 rural villagers from 10 regions throughout Thailand. Electrophoresis of 10 gm percent hemolysates extracted with toluene were performed at 4°C using vertical starch gel electrophoresis for 16 hours at 5 V/cm. Following this the gel slab was sliced with a wire and one-half was incubated for 6-10 hours at 37°C with a substrate containing 20 mg of 6-phosphogluconate, 10 mg of NADP, 4 mg of phenozine methosulfate, 150 mg of magnesium chloride, and 10 mg of nitro-blue tetrazolium, all in 100 ml of 0.5 M Tris buffer at pH 8.6. The other half of the gel was incubated with the same substrate mixture containing glucose-6-phosphate instead of 6-PG.

In the red cells of all of the males with detectable G-6-PD activity the electrophoretic mobility was identical to that of the B type of Caucasians. The electrophoretic patterns of the deficient males showed virtually no enzymatic activity. No other G-6-PD variants were detected.

The predominant phenotype of 6-phosphogluconate dehydrogenase (6-PGD) was the original type I of Fildes and Parr or the type A of Bowman et al. and Dern et al. In this phenotype, in addition to the anodally migrating major electrophoretic component, there are several more slowly moving minor fractions.

A cathodally (slowly) migrating component referred to as the C band by Fildes and Parr comprises only a few percent of the total enzyme activity. A smaller minor component which is visible only in very concentrated hemolysates migrates between the A and C bands. Another minor component migrates anodally to the major (A) band. Another minor component in the A phenotype, migrating cathodally to the C band, is usually obscured by Hb A but is clearly visible in hemolysates from homozygotes for Hb E.

The AB phenotype (A/B) is commonly seen in the Thai samples as a reduction in the intensity of the A band, with a somewhat heavier band (B) midway between the A and C bands and an increase in the intensity of the C band compared with the minor amount present in the A phenotype. The ratio of A:B:C was approximately 30:50:20. The B phenotype was observed in one specimen as heavy B and C bands and persistence of a small amount of activity in the region of the A band.

In five individuals a heretofore undescribed variant form of 6-PGD, referred to as the Thai variant, was observed. In this the A band is reduced in intensity. Anodal to this are two major bands, the cathodal one overlaying the fast minor component. These three bands are equidistant and their relative ratios are approximately 30:50:20, respectively. In addition, the C band is apparently split into two components of equal intensity, one with the mobility of C and one lying between the C and A bands with the mobility of B.

The frequency of G-6-PD deficiency in males is equivalent to gene (Gd) frequency. The highest frequency of Gd found was in Northeastern Thailand, 17.48% and 15.78% in two specific areas. In Southern Thailand, especially about midway down the Malayan Peninsula, the frequency of Gd was significantly lower, being only 2.83%.

The frequency of the A/B phenotype of the autosomal 6-PGD system did not differ in males and females in any of the ten locations. The mean frequency was 8.7%. The highest frequency, about 12%, was found in Central and Northcentral Thailand. From there it falls off to about 7% in the northeastern part of the country, to 3.7% in Chiengmai in North Thailand, and 8.1% in the far south.

Only one example of type B was detected. The cumulative number expected in the entire study was 6.2. In no single area was the departure from the Hardy-Weinberg equilibrium significant.

No samples deficient in 6-PGD activity were found.

Summary and Conclusions.

3185 subjects from 10 provinces throughout Thailand were sampled. In 1577 males the frequency of G-6-PD deficiency was 11.98%. In the far south the gene frequency was 2.83%; in the remainder of the country the frequency did not vary significantly about a mean of 13.76%. The deficiency is of a severe type. The G-6-PD of all non-deficients had the electrophoretic mobility of Type B.

The mean frequency of the A/B phenotype of 6-PGD was 8.47%. The maximum frequency was in Central and South Thailand, with a decline to the north and northeast.

A variant form of 6-PGD, referred to as the Thai variant, was found in which two additional electrophoretic components migrate anodally to the normal A band, confirming that the molecule is at least a dimer. The hypothesis is advanced that erythrocyte 6-PGD is determined by two genetic loci, only one of which is translated in leucocytes.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA6510	CSCRD-103

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

CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 124

10b. PRIOR NUMBER/CODE:

TITLE: (U) ENCODING OF PLANAR GRAPHS FOR CHEMICAL STRUCTURE RETRIEVAL CN

SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
700 ALGEBRA, PATHEPAT	11 66	NA	OTHER DA

PROGRAM, METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	11 67			
	a. NUMBER DA MD 49 193 67 G9218	PRIOR FY 68	0	11
c. GRANT	c. TYPE Y.GRANT d. AMOUNT $15,720	CURRENT FY 69	C	0

19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
WALTER REED ARMY INST OF RES	NAME: UNIVERSITY OF TORONTO
ADDRESS: WASHINGTON D C 20012	ADDRESS: ONTARIO, CANADA
	INVESTIGATORS PRINCIPAL: GOTLIEB, C. C.
	ASSOCIATE: JACOBUS, D. P.
a. INDIV. MERONEY, COL W. H.	
TEL. 202-576-3551	TEL. 202-576-2280 TYPE UB

TECHNOLOGY UTILIZATION	22. COORDINATION
MATHEMATICS ALGEBRA	NA

KEYWORDS: MATRIX THEORY, ALGEBRA, MATHEMATICS.

(U) TECH OBJECTIVE - A METHOD OF ENCODING PLANAR GRAPHS WHICH IS MINIMAL IN LENGTH HAS PREVIOUSLY BEEN DESCRIBED BY LIST CODING AS IDEAL FROM A COMPUTER POINT OF VIEW. IT IS NOT RECOGNIZABLE TO A CHEMIST. THE TECHNICAL OBJECTIVE OF THIS WORK IS TO DEVELOP A MECHANISM FOR DECODING THIS COMPUTER NOTATION.

(U) APPROACH- THE PARENTHESIS CODE IS TO BE TRANSLATED BACK INTO COORDINANTS SO AS TO CONSTITUTE A (PICTURE). THE TECHNIQUE INVOLVES THE ROUTING OF THE CHEMICAL GRAPH IN A PLANE STARTING FROM AN EDGE RATHER THAN FROM THE CENTER OR CENTERS OF THE GRAPH.

(U) PROGRESS - JUL 67 THRU JUN 68 THE CONTRACT WITH THE UNIVERSITY OF TORONTO IS CONCERNED WITH THE PROBLEM OF ISOMORPHISM WITH THE SPECIFIC OBJECTIVE OF ATTEMPTING TO STAY WITHIN THE PARENTHESIS-BRACKET CODE FOR REPRESENTATION OF CHEMICAL STRUCTURES AND YET SO-MANIPULATE THE CODE AS TO OBTAIN A TREE PERMITTING EASY CODING AND DECODING OF THE NOTATION TO CONVENTIONAL CHEMICAL PROJECTIONS. THE THEORETICAL STUDY ON THE MATHEMATICAL PROPERTIES OF CHEMICAL DIAGRAMS CONTINUES TO BE OF PROFOUND IMPORTANCE FOR THE COMPUTER PROGRAMS INVOLVING THE MANIPULATIONS OF CHEMICAL STRUCTURES. SOME OF THESE TECHNIQUES ARE TO BE INCORPORATED IN THE PROGRAM FOR THE NEW URAIR COMPUTER. DURING THE PAST YEAR ONE MASTERS THESIS, ENTITLED REDUCTION OF PLANAR GRAPHS HAS EMERGED FROM THE WORK. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
a. COMSEC OR COMSEC RELATED ☐ b. NOT RELATED ☒		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.

DD FORM 1 JAN 65 1493m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 124, Encoding of planar graphs for chemical structure retrieval

Investigators.
Principal: Dr. C. C. Gotlieb
Associate: Dr. A. H. Lehman

Description.

The purpose of this work is to determine the theoretical considerations in the computer encoding of chemical structures. This work is most important in order to be sure that we do not build a chemical information system which is mathematically unsound. There are several technical areas in which problems exist. One area involves the development of a relatively short notation for the representation of chemical structures. This means the development of a code which is close to the theoretical limits previously determined to be necessary for the unequivocal representation of structures. The code presently under development is "the parenthesis-bracket code."

The second problem is then to determine if one graph is isomorphic with another, i.e., in terms of chemistry, is one molecule the same as another.

The third problem is a piece of one graph embedded in a second graph. This is known as the problem of inclusion. Appreciable progress in the mathematical aspects of this problem continues to be made. The problem has been translated to the series of Ph.D. and Masters candidates who have produced studies on various portions of the work. The most recent contribution is work by Dr. Derek Corneil on the problem of graph isomorphism. This program which is mathematically sound may well be useful in determining chemical identity.

Progress.

The contract with the University of Toronto is concerned with the problem of isomorphism with the specific objective of attempting to stay within the parenthesis-bracket code for representation of chemical structures, and yet so manipulate the code as to obtain a tree permitting easy coding and decoding of the notation to conventional chemical projections. The theoretical study on the mathematical properties of chemical diagrams continues to be of profound importance for the computer programs involving the manipulations of chemical structures. Some of these techniques are to be incorporated in the program for the new WRAIR computer. During the past year, one masters thesis entitled "Reduction of Planar Graphs" has emerged from the work.

Summary and Conclusions

The development of sound procedures for the handling of chemical structures on the new machine is of fundamental importance. During the past year significant progress has been made in developing an algorithm which will be useful for the programming connected with chemical identity.

Publications

Gotshalks, G. J., Reduction of Planar Graphs. Masters thesis.

Corneil, D. G., Graph Isomorphism. Ph.D. thesis.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA CA6529	CSCRD-103

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

10a. CURRENT NUMBER/CODE
61130011 3A013001A91C 00 127

10b. PRIOR NUMBER/CODE

11. TITLE (U) STUDY OF RETRIEVAL OF BIOLOGICAL RESEARCH INFORMATION AS SUPPORT FOR LABORATORY INVESTIGATORS

12. SCIENTIFIC OR TECH. AREA
002600 BIOLOGY 004200 COMPUTERS

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
08 66	NA	OTHER DA

16. PROCURE. METHOD: B. CONTRACT

17. CONTRACT/GRANT: a. NUMBER DA 49 193 MD 3039; b. TYPE A.FPF; c. AMOUNT $145,437; d. DATE 08 66

18. RESOURCES EST.	a. PROFESSIONAL MAN - YEARS	b. FUNDS (In thousands)
PRIOR FY 68	1	76
CURRENT FY 69	0	6

19. GOVT. LAB/INSTALLATION/ACTIVITY
NAME WALTER REED ARMY INST OF RES
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INVESTIGATORS PRINCIPAL PARKINS, P. V.
ASSOCIATE JACOBUS, D. P.
TEL. 215-LO 9-1100 TYPE UN

21. TECHNOLOGY UTILIZATION UNIVERSITIES
PHARMACEUTICAL 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.

(U) APPROACH- BIOLOGICAL ABSTRACTS PROVIDES COVERAGE OF APPROXIMATELY 1CC,0CO ABSTRACTS PER YEAR. MACHINE TAPES FOR THE INDEX TERMS, AUTHOR, ORIGINAL CITATIONS AND MICROFILM OF 9C0,0CO ABSTRACTS ARE AVAILABLE. WRAIR PERSONNEL REQUEST ABSTRACTS BY AUTHOR, SELECTED WORDS, SUBJECTS, OR BROAD AREA OF INTEREST. THESE BITS OF INFORMATION ARE ENTERED INTO THE COMPUTER, WITH A PRINTOUT PROVIDED OF ALL ABSTRACTS PERTAINING TO THE USERS REQUEST. HARD COPIES OF THESE ABSTRACTS ARE PROVIDED THE REQUESTOR WITHIN 24 TO 48 HOURS.

(U) PROGRESS - JUL 67 THRU JUN 68 PROJECT EXPERT HAS NOW BEEN ADOPTED BY SEVERAL ELEMENTS OF THE U. S. ARMY MEDICAL R+D COMMAND, NAMELY, WALTER REED ARMY INSTITUTE OF RESEARCH, R+D HQ AND LETTERMAN GENERAL HOSPITAL. PUBLICATIONS HAVE NOW BEEN WRITTEN TO DESCRIBE THE SYSTEM. THE ORIGINAL SEARCH TECHNIQUES WITH OUTPUT IN THE FORM OF ABSTRACTS ARE OF PROVEN USEFULNESS. THE ABSTRACT COLLECTION IS NOW APPROXIMATELY 90C,000. LABORATORY INVESTIGATORS CONTINUE TO DEMONSTRATE LITTLE INTEREST IN SEARCH TECHNIQUES. THE SPECIFIC HYPOTHESIS CONCERNING THIS LACK OF INTEREST IS THE RELATIVELY (3DAYS) SLOW RESPONSE IF SEARCH STRATEGY IS DELIVERED SINCE THAT STRATEGY USUALLY IS INCLUDED WITH THE ABSTRACTS ANSWERING A QUESTION. A DATA CELL CAPABLE OF HANDLING THE EXISTING FILE OF INDICES HAS BEEN DELIVERED TO BIOLOGICAL ABSTRACTS THEREBY MAKING POSSIBLE THE DIRECT TRANSMISSION OF SEARCH STRATEGY WITHIN AN HOUR OF RECEIPT OF THE QUESTION. THE FUTURE RESEARCH PLAN IS TO DELIVER TO WALTER REED ARMY INSTITUTE OF RESEARCH AN OUTPUT DEVICE CAPABLE OF DISPLAYING THE DATA CELL INDICES THEREBY ENABLING THE WALTER REED ARMY INSTITUTE OF RESEARCH INVESTIGATORS TO SELECT THEIR OWN STRATEGY. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.
FY - 1	

DD FORM 1 JAN 64 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 127, Study of retrieval of biological research information as support for laboratory investigators

Investigators.
Principal: Phyllis V. Parkins
Associate: Louise Schultz

Description.

The objectives of this study are multiple. The first is to provide abstract services for WRAIR and other AMEDS investigators. As a result of such studies to determine the "literatureness" of abstracts as well as to provide measurable information on the future role of abstracts and what investigators will use.

Progress.

Progress to the present time has involved the establishment of a system which appears to be successful if one judges success either by the service provided, the influence on other needs, such as Interlibrary Loans, or a subjective survey among the people who have used the system. This report attempts to determine the degree of satisfaction of requesters in a custom literature search service in the biological sciences from the patterns of system usage over a 33-month period. Studies of users and usage of an information system (1-3) commonly depend on questionnaires, interviews, and diaries through which the user reveals his information needs, use of the system, or degree of satisfaction with its performance. BioSciences Information Service (BIOSIS) has been operating and developing an information system (4) since May 1965, to the evaluation of which it could apply none of these measurement methods. This study suggests that performance evaluation can be inferred from empirical operations data gathered routinely and not influenced by user subjectivity (in the data-gathering phase).

The System, Its Environment, And Its Users

BioSciences Information Service of Biological Abstracts (BIOSIS) functions as both a secondary recorded-medium channel, in publishing Biological Abstracts and sister publications, and a type of information-center channel, in offering custom abstracts retrieval service (5). Since its founding in 1927, Biological Abstracts has published approximately two million abstracts and citations of primary journal articles in the biological sciences (6). Of these, indexes to nearly one million are available in machine form and the corresponding abstracts are on microfilm for duplication by a reader-printer.

To exploit this data base, an essentially manual retrieval service was initiated in May 1965 under the sponsorship of Walter Reed Army Institute of Research (WRAIR) (7), which has developed in the degree of computer support and has expanded to serve the research unit at Letterman General Hospital, San Francisco, and the U. S. Army Medical Corps Research and Development Command, Scientific and Technical Information Office (STINFO). The latter facility has not been included in this study because data are not available as to the identity of requesters, inasmuch as requests are entered by an intermediary in the 'name' of the facility.

The requester population at the using installations consists of both military and civilian personnel. There is a constant turnover of the former due to termination of duty tours. Individuals presently on staff at WRAIR were identified from a current directory, allowing comparisons to be made between those who still had access to the system at the end of the study period and those who had left WRAIR between the time of system usage and the end of the study period. This information was not available for requesters at Letterman General Hospital or U. S. Army Institute of Dental Research. Neither are precise data available on the maximum number of individuals having the opportunity to use the system during the period, although we judge the number to be in the order of 1500, including all technical and administrative personnel. Work missions vary but include "bench research," evaluation of technical merit of proposals seeking funding support, and participation in or planning of special scientific short courses.

The interface between user and system consists of electrowriter-dataphone terminals located at the WRAIR library* and the Research and Development division of BIOSIS. An individual desiring to use the system obtains a dial-a-card from the librarian to establish

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* Terminals are also located at the other user installations mentioned.

communications with BIOSIS through telephone lines. He writes his inquiry message in natural language, in his own handwriting, on the electrowriter.

Receipt of the written message is usually monitored by experienced biologists at BIOSIS. When necessary, the BIOSIS 'search strategist' may negotiate with the requester, either in writing or by voice communication, to clarify ambiguities in the question or correct for electronic disturbances in the transmission. As discussed by Cavanaugh (8), this type of dynamic interface, in which no constraints are imposed on dialogue between user and system operator is superior to one that offers only yes/no responses or one that requires the user to interpret the system responses. BIOSIS personnel are thus better able to formulate an effective search strategy.

This strategy is the plan by which BIOSIS personnel identify abstracts most likely to contain the information desired by the requester. It consists of

a. designation of files to be searched (keyword, subject, taxonomic and/or author indexes);

b. distribution of search tasks between computer and humans; and

c. assignment to appropriate search personnel (biologists and/or clerical).

Abstracts meeting search specifications are reproduced

from microfilm on a reader-printer and screened by biologically trained personnel as to their pertinence to the query. Those selected are then mailed to the requestor.

Data about each transmission and response are on punch cards for machine processing of operations reports. The information includes the user's name, department, date of transmission, transmission number, and number of abstracts sent. A "transmission" is defined as a communication from user to the system regardless of the number of questions (or "search tasks") encompassed (9). Operations records, including for this report the calculation of intervals between transmissions, are based on number of working days per year rather than on calendar days; e.g., one year = approximately 251 days.

Usage Patterns and Satisfaction Implications

This report considers the number of transmissions per user, the length of intervals (in working days) between an individual's transmissions, and number of abstracts sent per transmission. Information was correlated with the individual's department and/or installation, to examine implicit differences in user needs and satisfaction with the service based on his scientific discipline.

Between the inception of this information service and 31 January 1968, BIOSIS had processed more than 1000 transmissions from 416 different individuals from WRAIR, USAIDR and Letterman General Hospital Research Unit. Figure 1 plots the number of requests in quarterly intervals. The high initial activity can be accounted for by the novelty of the system and the fact that, by service contract definition, a correspondence existed between a "transmission" and a "search task." Subsequent to the first year, multiple requests per transmission were permitted. Excluding the first four quarters, the average is 85 transmissions per quarter with a standard deviation of 13 and a mean deviation of 11.

Completely without formal publicity or encouragement by WRAIR managers to use the system, first-time use has apparently increased steadily since the second quarter of 1966, as graphed in Figure 2. We can assume not only that total system usage levels are maintained in an environment of changing patrons (Figure 1) but also that "satisfied" users have mentioned this service to their colleagues, especially to new arrivals at the installation.

Another indication of user satisfaction might be his repeated use of the system. Of the total sample,

48 percent of the requesters initiated more than one transmission, the average being 2.5 transmissions per individual, 2.7 for WRAIR personnel. Figure 3 also illustrates the percentage of total users in each multiple-transmission group.

In Figure 4, the pattern of access is plotted according to whether the individual is known to have been on the WRAIR staff at the end of the study period (labelled "WRAIR PRESENT"), apparently had left WRAIR (labelled "WRAIR NOT PRESENT"), or is from another installation and whose status is unknown (labelled "NON-WRAIR"). Notice that the pattern for both of the latter categories exhibits a definite cut-off trend. However, system drop-out for multiple-transmission users who are known to have been on the WRAIR staff at the close of the study period appears, in contrast, to approach a threshold, implying a measurement of satisfaction. That is, if the user re-enters the system once, the probabilities are 2 out of 3 that he will re-enter again and 1 out of 3 that he will re-enter four or more times. One out of seven users continuing to have access to the system develop a pattern of reuse averaging one transmission per quarter. Ten individuals at WRAIR used the system nine or more times. Of these, four initiated more than 20 transmissions. Such behavior must be interpreted as satisfaction with system performance.

If multiple re-entry can be interpreted as a measure of satisfaction with system performance, single-entries--at least by any individual known to have been on the WRAIR staff at the end of the study period--must be examined as a measurement of dissatisfaction. Only 34 percent of the non-repeaters fall into the "WRAIR PRESENT" category (Figures 3 and 4). In Table 1, grouped by calendar quarter in which the inquiry was transmitted, are the number, and percentage of the total represented by that number, of one-time requestors.

For those known to have continuing access to the system, observe the minimum of about three percent drop-out per quarter and the maximum of 13 percent. Assuming the minimum to be the maximum tolerable, we may consider the higher initial drop-out as an 'adjustment' phase of operation upon which performance was improved during calendar 1966. Adjustment also encompasses the effects of the contract definition of a "search" mentioned earlier and discussed in detail in reference 9.

The unprecedented drop-out rate of the first three quarters of 1967 is influenced by (at least) the fact that re-entry occurs at some interval after first-time entry. That is, based on analysis of the interval

between first and second entries for multiple-transmission users, we can correct the drop-out data. If the requester has not re-entered by the end of the same quarter, the probabilities are 1:3 that he will re-enter subsequently; by the end of the subsequent quarter, they drop to 1:6; by the end of the third quarter, to 1:10; by the end of the fourth quarter, to 1:14; by the end of the fifth quarter, to 1:20.

Thus, one of the 10 drop-outs from the first quarter of 1967 may still be expected to re-enter (after the end of the study period), one of the nine from the second quarter, two of the ten from the third quarter, and two of the five from the fourth quarter. Although the resulting "corrected" drop-out rate for the fourth quarter is acceptable as an indication of improving system performance (which continues into 1968), the data for the first three quarters persist as a barometer of failure of the system to meet the needs of first-time requesters during that period. Jacobus (9) detects the same failure in data on inter-library loan requests by potential system users. The deadline date for submission of this paper precludes publication of data for 1968; data through 30 June 1968 will be available for discussion at the meeting, however.

As implied above, the criterion for the performance is modified by the behavior of "satisfied" or multiple-entry users. Figure 5 illustrates the average intervals between transmissions, categorized on the basis of the total number of transmissions made by the user. These data, plus the shortest interval and longest interval between successive transmissions and between first and last entry, among those by individuals entering the system twice or more, are provided in Table 2.

In the graph of averages, we account for three factors:

1. for a fixed-length period, the length of interval between events perforce decreases as the number of events increases;

2. an information need, satisfaction of which requires exhaustive exploitation of a given data base (extremely large but growing only at about one percent per month), arises at some interval, called here a "nominal task interval" for any one individual; and

3. user confidence in system performance motivates re-entry at intervals shorter than the "nominal task interval" perhaps, at least in part, because the task is accomplished or redirected more quickly with information service support.

The comparative stability of interval-length for the six-transmission user leads us to consider these data the basis for a norm. That is, we define a "nominal task interval" as greater than 60 working days (approximately 3 calendar months) but less than the 126 working days between first and second entries by the two-transmission user. Further analysis is being made of comparable data for those users known to still have access to the system. However, the small number of individuals in the sample for six or more transmissions may be subject to too many variables of professional competence, sophistication in use of information services, work mission, etc., to justify drawing more incisive conclusions regarding differences in length of intervals between usage.

The final set of data presented here is given in Table 3, to examine the effects on user satisfaction (i.e., usage) of correspondence between his scientific discipline and BIOSIS file characteristics. For each research division of WRAIR are listed the total number of individuals who have transmitted at least one inquiry message, the number and percent of the total who are known to still have access to the system, and for each of these groups the average number of transmissions and average number of abstracts delivered per transmission. In general, the principal work mission of these individuals is laboratory research.

BIOSIS' policy for coverage of the biological literature emphasizes research aspects and de-emphasizes clinical and/or practice-oriented literature. The data indicate comparatively high continued usage by the divisions of Medicinal Chemistry, Neuropsychiatry, and Experimental Pathology. The Nursing division, as might be expected, exhibits the lowest usage. Data on the Biochemistry division apparently indicate recent separation of previously active users from the using installation (low median number of days those 'present' have been using the system).

The average number of abstracts delivered per transmission reflects the precision with which the inquiry was posed or else the effects of coverage policy. Excluding the single transmission by a Nursing division user, the high of 195 abstracts per transmission from the Medicinal Chemistry division results from a preference among this group to browse through broad responses. Satisfaction of Experimental Pathology and Neuropsychiatry divisions users, exhibited by the high transmission rates, may also be attributed to a preference for relatively precise responses indicated by the number of abstracts sent.

Acknowledgements

The authors especially thank Mrs. Amie Rumsey of BioSciences Information Service for the illustrations in this report and Mrs. Evelyn Neuschatz, Librarian at WRAIR, for kindly supplying the directory of current personnel. They are also indebted to all other members of BIOSIS R&D staff and to Dr. Jacobus and the WRAIR staff for suggestions and data.

References

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8 Cavanaugh, J. M. A., "Some Considerations Relating to User-System Interaction in Information Retrieval Systems", Fourth Annual National Colloquium on Information Retrieval, Albert B. Tonik, ed., International Information Inc., Philadelphia, 1967, 119-126.

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Abstract

A study was made of the usage of the BioSciences Information Service (BIOSIS) custom literature search service in order to measure, inferentially, user satisfaction. Data are included on single- and multiple-entries into the system, patterns of system drop-out, intervals between requests, and volume of system responses. The effect on these parameters of the user's scientific discipline, and continuing access to the system, is examined.

User satisfaction was inferred from re-entry behavior; however, the frequency and number of re-entries per user also appears to correlate with a "nominal task interval" and with term of stay at the using installation.

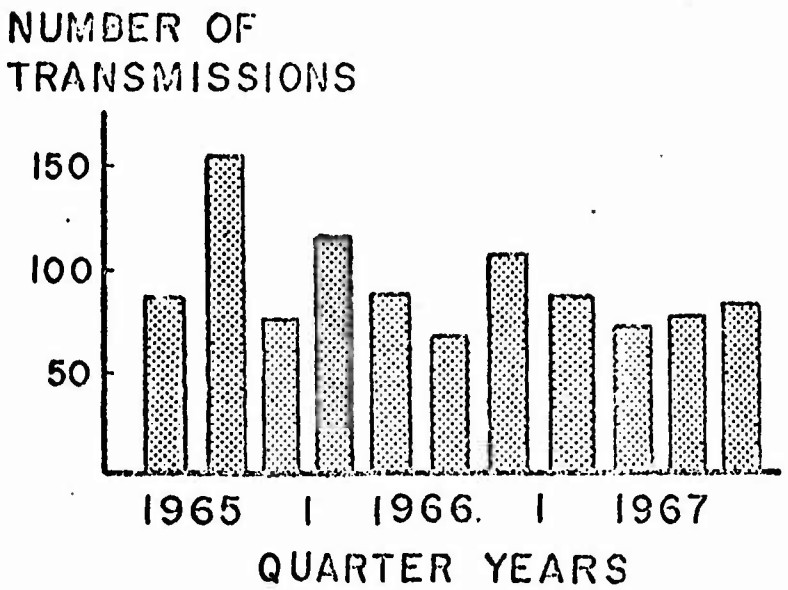

FIGURE 1. Transmissions to BIOSIS Search Service by Calendar Quarter.

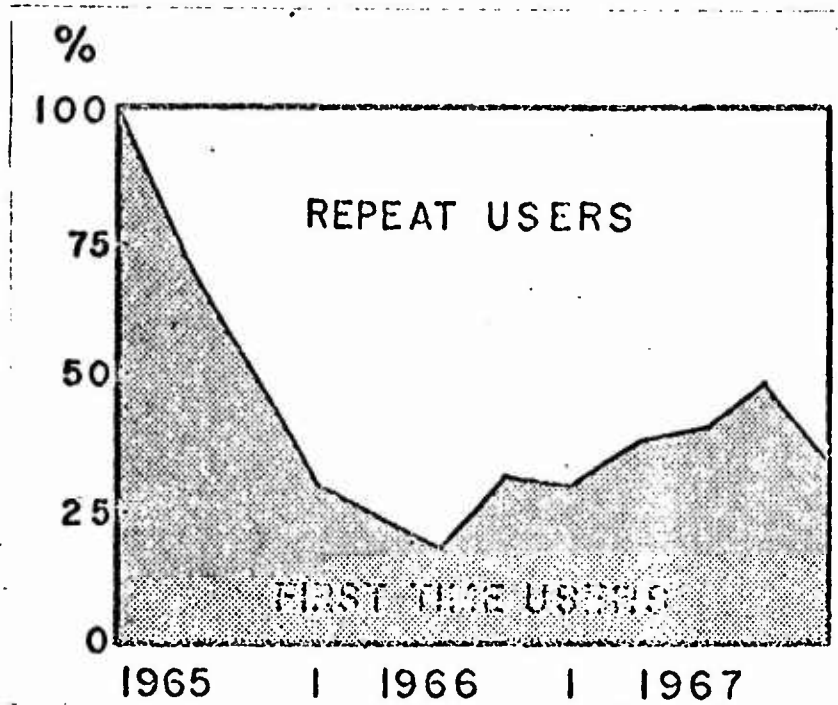

FIGURE 2. Proportion of First-Time and Repeat Users of BIOSIS Search Service.

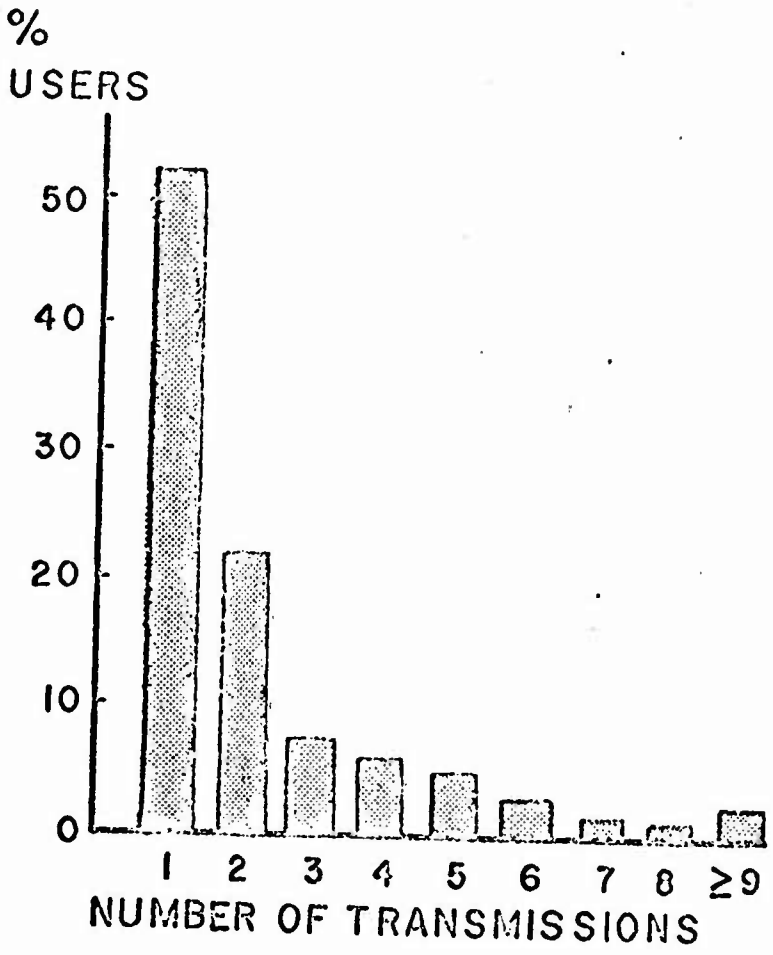

FIGURE 3. Percentage of Users Transmitting One or More Inquiries.

YR	QTR	TOTAL TRANS	FIRST TRANS	ONE-TIME REQUESTORS NUMBER			PERCENTAGE		
				P	G	N	P	G	N
65	2	88	64	6	15	1	6.8	17.1	1.3
	3	155	85	8	27	2	5.2	17.4	1.3
	4	75	28	5	5	0	6.7	6.7	0.0
66	1	117	27	3	4	2	2.6	3.4	1.7
	2	88	16	3	3	1	3.4	3.4	1.1
	3	69	23	4	6	0	5.8	8.7	0.0
	4	107	36	4	12	5	3.7	11.2	4.7
67	1	98	37	10	9	8	13.1	9.2	8.2
	2	71	28	9	6	6	12.7	8.5	8.5
	3	77	35	10	2	8	13.0	2.6	11.3
	4	81	29	5	5	14	6.2	6.2	17.3

P=WRAIR PRESENT G=WRAIR NOT PRESENT
N=NON-WRAIR

TABLE 1. System Drop-Out Data

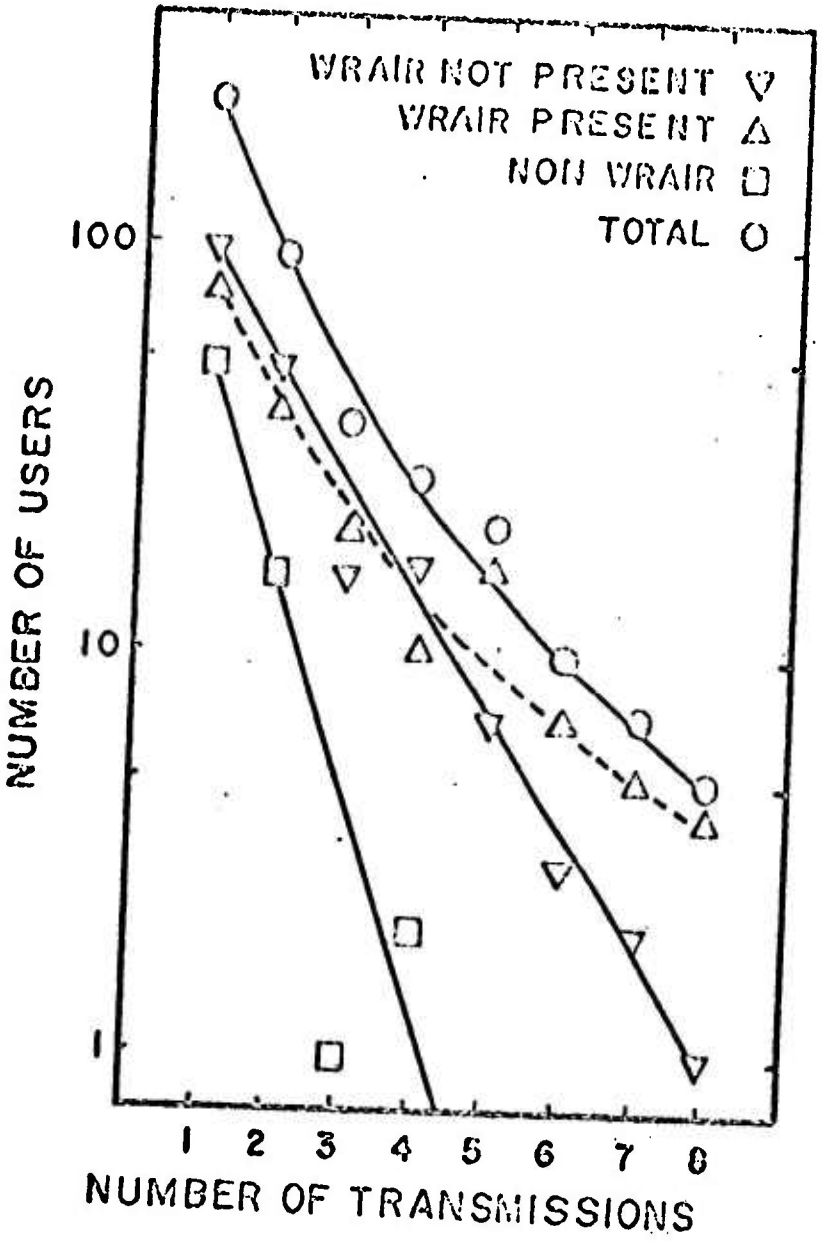

FIGURE 4. Difference in Repeat Usage as a Function of Continued Access to the System.

TRANS	FIRST			SECOND			THIRD			FOURTH			FIFTH			SIXTH			ALL		
	S	AVE	MAX	S	AVE	MAX	S	AVE	MAX	S	AVE	MAX	S	AVE	MAX	S	AVE	MAX	S	AVE	MAX
2	1	126	648																1	126	648
3	1	72	547	3	135	485													6	207	559
4	1	48	375	1	52	263	2	104	460										12	202	503
5	2	52	272	4	49	168	4	93	342	3	106	302							53	300	596
6	2	37	107	6	60	187	5	61	174	5	65	211	1	67	288				34	285	597
7	1	22	79	1	39	115	3	72	219	1	82	315	2	109	277	11	61	107	113	385	653
8	6	61	127	1	43	126	1	18	45	4	52	122	19	52	99	71	129	157	194	386	608

S= SHORTEST

TABLE 2. Length of Interval Between Transmissions for each Access by Multiple-Transmission Users.

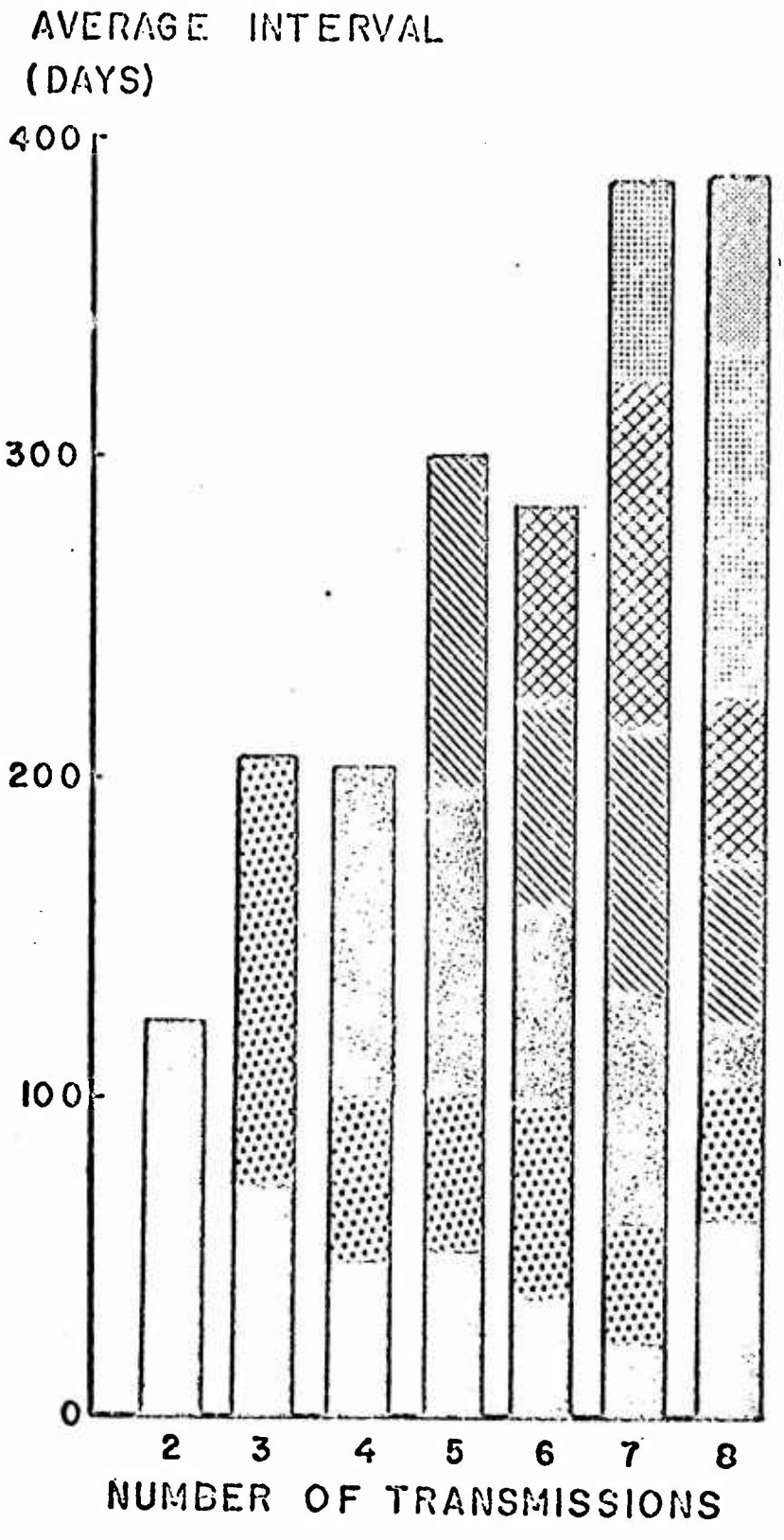

FIGURE 5. Average interval between transmission for multiple-transmission users. (Intervals graphed in order of occurrence.)

DIVISION NAME	TOTAL USERS FROM DIV.	USERS PRESENT		TOTAL USERS		USERS PRESENT		DAYS IN SYSTEM	
		NO.	%	TRANS/ USER	ABS/ TRANS	TRANS/ USER	ABS/ TRANS	AVE.	MED.
BIOCHEMISTRY	8	3	38	4.9	87	1.0	8*	143	62
COMM.DIS.AND IMMUNOLOGY	63	34	54	1.8	161	1.9	139*	404	524
DENTISTRY	29			1.4	30				
EXP.PATHOLOGY	14	10	72	4.4	59	4.7	51	438	379
MEDICINE	66	36	55	3.6	158	3.5	106	375	408
MED.CHEMISTRY	25	15	60	4.3	222	5.5	195	367	323
NEUROPSYCH.	32	14	44	3.4	67	5.3	50*	475	550
NUCLEAR MED.	25	13	52	2.6	111	2.7	62	407	579
NURSING	6	1	17	1.2	66	1.0	380	655	655
PREVENT.MED.	17	6	35	2.0	97	3.1	142	356	420
SURGERY	28	11	39	2.4	106	2.6	40	213	201
VETERIN.MED.	20	8	40	1.9	204	2.4	140	335	373
AVERAGES	28	13	47	2.9	114	3.1	119	379	
MEDIAN	25	11	44	2.5	102	2.7	106	375	

*=ONE OR MORE 'NEGATIVE'. NO ABSTRACTS SENT

TABLE 3. Variation in Usage by Division (Scientific Discipline)

Summary and Conclusions.

Rather than measuring performance relative to percentages of "appropriate" and "inappropriate" items elicited from a file in response to designed inquiries, BIOSIS is gauging the performance of a developing retrieval system by the behavior of a significant number of users over a significantly long period and in a significant number of actual inquiry events. The prediction model is then the standard for evaluating the effect of operational changes to the system.

The WRAIR staff, judging by the effect of Interlibrary Loans and a survey of interested users, have indicated that they believe the system as it now exists is effective especially in the support of those investigators who have broad, complex interests.

Publications.

Jacobus, D. P., et al. Direct User Access to the Biological Literature Through Abstracts: A Cooperative Experiment in Customized Service. BioScience, 16:599-603, 1966.

Jacobus, D. P., et al. Experience with a Mechanized Biological Information Service. Abstract. American Chemical Society, 13 Sep 1967.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6529	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 31 03 69	"U" WHY "U"	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 128

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) TRANSMISSION, CONTROL AND TREATMENT OF INFECTIOUSDISEASES OF MILITARY IMPORTANCE IN EQUATORIAL ASIA

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CMPT. COMPL. DATE	15. FUNDING AGENCY
C1C100 MICROBIOLOGY	09 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
A. GRANT	a. NUMBER DADA17-C-C-C9253 b. DATE 09 66 c. TYPE Y.GRANT d. AMOUNT $10,000	PRIOR FY 68	0	10
		CURRENT FY 69	0	10

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

RESP. INDIV: MERONEY, COL W. H.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: US ARMY MED RSCH UNIT
ADDRESS: INST MED RSCH
KUALALUMPUR MALAYSIA

INVESTIGATORS PRINCIPAL: OMAR-AHMAD, U.
ASSOCIATE: RAPMUND, LTC G.
TEL: 202-576-3061 TYPE: ZT

21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NA

23. KEYWORDS: TROPICAL DISEASE.

24.

(U) TECH OBJECTIVE - TO FACILITATE CERTAIN STUDIES ON DISEASE IN MALAYSIA.

(U) APPROACH- THIS IS A SUPPLEMENT TO AN EXISTING CONTRACT TO PROVIDE ILIR FUNDS TO THE COMMANDING OFFICER OF THE UNITED STATES ARMY MEDICAL RESEARCH UNIT IN KUALA LUMPUR, MALAYSIA.

(U) PROGRESS - JAN 68 THRU MAR 68 TWO ABORIGINAL COMMUNITIES HAVE BEEN SELECTED FOR A LONGITUDINAL STUDY OF THE BACTERIAL FECAL FLORA OF ISOLATED POPULATIONS. IN AN ATTEMPT TO DETERMINE IF THE SO-CALLED R FACTOR EXISTED PRIOR TO THE ANTIBIOTIC ERA, MORE THAN 1400 ISOLATES OF BACTERIA HAVE BEEN OBTAINED FROM THE FECAL FLORA OF 128 PERSONS IN FOUR ISOLATED COMMUNITIES IN EAST MALAYSIA. THESE VILLAGES ARE NOT KNOWN EVER TO HAVE RECEIVED ANY ANTIBIOTICS WITH THE EXCEPTION OF 19 INJECTIONS OF PENICILLIN IN 1959. THE ANTIBIOTIC SENSITIVITY OF THESE ORGANISMS IS BEING DETERMINED. TESTS WILL BE MADE TO FIND IF MULTIPLE RESISTANT BACTERIAL ARE ABLE TO TRANSFER RESISTANCE TO SENSITIVE STRAINS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.

DD FORM 1 JAN 68 1493m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 25 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 128, Distribution of intestinal bacterial pathogens in Malaysian aborigines

Investigators.
Principal: Ungku Omar-Ahmad, M.D.
Associate: LTC Garrison Rapmund, MC
CPT C. E. Davis, MC

Description.

The principal objective of this investigation, which is one part of a larger study on infectious diseases of military importance in equatorial Asia, is to study the etiology and transmission of infectious diarrhea in certain Malaysian populations.

Progress.

This investigation was started in July 1967 and has been concerned thus far with organizing the study groups and collecting base-line information. Sixteen communities have been selected for study; 5 are located in deep jungle and 11 are in less isolated fringe areas. All inhabitants have been sampled at least once. Enteric pathogens were cultured twice as frequently from the fringe communities as from the jungle. Ten percent of the inhabitants in the former area had positive cultures and 60% of these individuals complained of diarrhea. Shigella species accounted for over 60% of the enteric pathogens in both types of communities. Salmonella species were rare in the deep jungle communities but were responsible for 40% of the positive cultures in the fringe area.

In accordance with other reports in the literature, rectal swabs were superior for isolating Shigella species and fecal specimens were superior for salmonellae.

Six of the "fringe" communities had recently experienced mild diarrhea epidemics. Cultures were obtained from 224 persons, of whom 94 complained of diarrhea. Enteropathogens were isolated from 12 (12.5%) of the group of 94 persons. Ten of the isolates were Shigella spp, and the other two were Salmonella spp.

Aborigines requiring hospitalization are transported from all over Malaysia to a single hospital. It was desirable to study hospitalized diarrheal cases because community cross-infection might occur via hospital contacts. On one occasion 96 diarrheal patients were cultured. Of these 24 had enteropathogens: 10 Shigella spp; 10 Salmonella spp; and 4 E. coli. Some

indication of hospital-acquired infections has been seen, but thus far there is no evidence of community cross-infection via the hospital.

The enteropathogens isolated so far have been remarkable for their lack of antibiotic resistance. Indeed, only one multiply resistant organism has been found, an S. infantis which was isolated from 13 individuals at the hospital for aborigines. An increasing number of resistant isolates are being found in other parts of the country. In an attempt to obtain information on the question of whether "R" factor existed prior to the antibiotic era in Malaysia, more than 1400 isolates of bacteria from the fecal flora of 128 persons have been obtained from 4 isolated communities in East Malaysia (Sabah). These villages are not known to ever have received any antibiotics with the exception of a total of 19 injections of penicillin in 1959. The antibiotic sensitivity of the organisms is in the process of being determined. Tests will be performed to determine if any multiply resistant forms can transfer their resistance to suitable sensitive strains.

Summary and Conclusions.

a. Diarrhea is common in Malaysian aborigines and enteropathogens can be isolated with relative ease from both symptomatic and asymptomatic individuals.

b. Enteric pathogens were cultured twice as frequently from individuals in fringe communities as from those in the deep jungle.

c. Shigella spp represent over 60% of enteric pathogens in both types of communities. Salmonella spp were rare in deep jungle communities but made up 40% of positive isolates in fringe communities.

d. Only one multiply resistant organism has been found thus far. This is an S. infantis that was obtained from 13 hospitalized diarrhea cases.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 0A6530	CSGRD-103

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

10 a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 0C 129

10 b. PRIOR NUMBER/CODE:

11. TITLE: (U) RETRIEVAL OF DATA IN TROPICAL DISEASES BULLETIN

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY 004200 COMPUTERS	08 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	08 66			
B. CONTRACT	b. NUMBER DA-49-193-MD-3058	PRIOR FY 68	2	98
	c. TYPE A.FPF d. AMOUNT $187,560	CURRENT FY 69	2	95

19. GOVT. LAB/INSTALLATION/ACTIVITY

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20. PERFORMING ORGANIZATION

NAME: BIOLOGICAL ABSTRACTS
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PHILA PA 19104

INVESTIGATORS PRINCIPAL: PARKINS, P. V.
ASSOCIATE: JACOBUS, D. P.
TEL: 215-LO 9-1100 TYPE: UN

21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NA

23. KEYWORDS: INFORMATION PROCESSING, INFORMATION RETRIEVAL, MICROBIOLOGY.

24.

(U) TECH OBJECTIVE - TO PROVIDE, IN A READILY ACCESSIBLE FORM, ALL DATA CONTAINED IN THE TROPICAL DISEASES BULLETINS, VOLS 1-62.

(U) APPROACH- SEVERAL APPROACHES ARE BEING MADE TO DETERMINE HOW THIS DATA CAN BE MOST ECONOMICALLY OBTAINED, I.E., FROM EXISTING INDEXES, FROM KEYWORDS IN TEXT AND TITLE, ETC.

(U) PROGRESS - AUG 67 THRU JUN 68 THE AUTHOR ENTRIES FOR ALL ISSUES OF THE TROPICAL DISEASE BULLETIN HAVE BEEN COMPLETED AS HAVE ALL OF THE SUBJECT INDICES. ALL BUT 10,000 TITLES HAVE LIKEWISE BEEN COMPLETED, ALTHOUGH THERE IS SOME ADDITIONAL PROOF-READING TO BE DONE ON THE COMPLETED TITLES. ALL MICROFILMING HAS BEEN COMPLETED. VOLUMES 1 - 44 ARE THEREFORE IN A FORM WHICH CAN BE CONVERTED INTO THE EXISTING BIOLOGICAL ABSTRACTS SYSTEM. THE FULL TEXT OF VOLUMES 45 - 63 HAVE LIKEWISE BEEN COMPLETED AND THEREFORE, IN ADDITION TO BEING AVAILABLE FOR INCLUSION IN THE EXISTING BIOLOGICAL ABSTRACTS SYSTEMS, ARE READY FOR FULL TEXT SEARCHING. FUTURE WORK VISUALIZES STAYING CURRENT WITH THE TROPICAL DISEASE BULLETIN AS IT IS PUBLISHED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In Thousands)	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 129, Retrieval of Data in Tropical Disease Bulletin

Investigators.
Principal: Mrs. P. Parkins
Associate: Miss L. Schultz, Biological Abstracts

Description.

The purpose of this investigation is two-fold. The first is to put into digital form all the information contained in the Tropical Disease Bulletin, Volumes 1 through 63, so that digital searching and digital display of appropriate abstracts can be achieved. The second objective is to develop capabilities for the handling of full text on a file of limited but yet significant size.

Progress.

The author entries for all issues of the Tropical Disease Bulletin have been completed as have all of the subject indices. All but 10,000 titles have likewise been completed, although there is some additional proofreading to be done on the completed titles. All microfilming has been completed. Volumes 1 through 44 are therefore in a form which can be converted into the existing Biological Abstracts system. The full text of Volumes 45 through 63 have likewise been completed and therefore, in addition to being available for inclusion in the existing Biological Abstracts system, are ready for full text searching. Future work visualizes staying current with the Tropical Disease Bulletin as it is published.

Summary and Conclusions.

This work is proceeding well. The speed of input has been such that the building of the full text system rather than an abbreviated text system appears feasible as a result of the detailed instructions generated for the typists. Further work on input variations is expected to lead to additional improvements. The availability of the Tropical Disease Bulletin in digital form is expected to improve WRAIR's coverage of both tropical diseases and geographic areas.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA6522	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY (a) PRIOR (b) THIS	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 07 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 170

12a. PRIOR NUMBER/CODE:

11. TITLE: (U) TRACE METAL CONCENTRATIONS IN BIOLOGICAL MATRICES

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
014000 RADIC + RADIATIC. CO6300 INORGANIC CHEMIST 003500 CLINICAL MEDICINE	10. 66	NA	OTHER DA

16. PROCUREMENT METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. TYPE NA	b. NUMBER NA c. DATE NA d. AMOUNT NA	FY 68	3	50
			CURRENT FY 69	3	50

19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME: WALTER REED ARMY INST OF RES	NAME: WALTER REED ARMY INST OF RES
ADDRESS: WASHINGTON D C 20012	ADDRESS: DIV OF NUCL MED WASHINGTON D C 20012
RESP. INDIV.: PERONEY, COL W. H.	INVESTIGATORS PRINCIPAL: MAHIN, LTC C. T.
TEL: 202-576-3551	ASSOCIATE: ANGEL, LTC C. R.
	TEL: 202-576-2211 TYPE: DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
RADIO + RADIATION CHEMISTRY CLINICAL MEDICINE	NA

23. KEYWORDS: TRACE ELEMENTS, HOMEOSTASIS, METABOLISM.

24.

(U) TECH OBJECTIVE - TO ESTABLISH MODERN METHODS OF QUALITATIVE AND QUANTITATIVE ANALYSIS FOR TRACE METALS IN HEALTH, DISEASE AND TOXICOLOGY WITHIN THE ARMY MEDICAL SERVICE.

(U) APPROACH- UTILIZING ATOMIC ABSORPTION SPECTROMETRY AND NEUTRON ACTIVATION ANALYSIS, CONCENTRATIONS OF TRACE ELEMENTS WILL BE DEFINED IN TERMS OF SITES OF ACTION AND CATALYTIC INTERACTION WITH SUBSTRATES. EMPHASIS WILL BE PLACED ON ANTAGONISTIC OR ADDITIVE EFFECTS WITH RELATED GROUPS OF ELEMENTS. INITIALLY, EFFORTS WILL BE LIMITED TO THE FOLLOWING BLOCK OF ELEMENTS- MANGANESE, COPPER, ZINC, SELENIUM, VANADIUM, COBALT AND IODINE.

(U) PROGRESS - JUL 67 THRU JUN 68 ADDITIONAL PROCEDURES HAVE BEEN DEVELOPED FOR ANALYSIS OF MANY TRACE METALS IN BIOLOGICAL MATRICES USING CHEMICAL METHODS, ATOMIC ABSORPTION SPECTROPHOTOMETRY AND NEUTRON ACTIVATION ANALYSIS. EMPHASIS HAS BEEN PLACED ON COPPER AND MANGANESE ANALYTICAL METHODOLOGY. ANALYTICAL TECHNIQUES HAVE BEEN UTILIZED IN THE STUDY OF TRACE METAL METABOLIC MALFUNCTION SUCH AS WILSONS DISEASE, VARIATIONS PRODUCED BY RADIOPROTECTIVE DRUGS AND LIVER MANGANESE DIFFERENCES IN MICE AS A FUNCTION OF AGE AND STRAIN. UTILIZATION OF THESE PROCEDURES IN SUPPORT OF DIAGNOSTIC AND CLINICAL SERVICES AT THE WALTER REED GENERAL HOSPITAL IS NOW WELL ESTABLISHED. FOR TECHNICAL REPORTS, SEE WRAIR ANNUAL PROGRESS REPORT, 1 JUL 1967 - 30 JUN 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ 2. CONSEC OR CONSEC RELATED ☒ 3. NOT RELATED		BR	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.

DD FORM 1498m JAN 68 REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 170, Trace metal concentrations in biological matrices

Investigators.
Principal: LTC Dorsey T. Mahin, MC
Associate: LTC Charles R. Angel, MSC; Robert T. Lofberg, Ph.D; Elvio A. Levri, MS; Mr. Billy G. Bass, BS; Ann R. Berman, BS; CPT Robert M. Donati, MC; Mary M. McLaughlin, MS; LTC LaWayne R. Stromberg, MC; Nuclear Medical Detachment, USAREUR: LTC Worthen Boyce, MC; Dept of Gastroenterology, WRGH.

Description.

The objective of this work unit is the quantitative determination of trace elements in defined biological systems. Comparative studies with other biochemical moieties are an important part of the work unit.

Progress.

1. Methodology.

In the following studies, several techniques have been employed. Considerable effort has been expended to improve our capability to make precise measurements of trace elements in biological systems. We have utilized neutron activation analysis, flame photometry, and atomic absorption techniques in these studies and have concentrated our attention on methods to measure iron, copper, magnesium, and zinc. We are now able to make reliable and reproducible measurements of these trace elements in biological systems to a precision of plus or minus three percent. We have performed these studies on the following biological materials: plasma, urine, gastric juice, saliva, tissue biopsy samples, fingernails, and hair. A method has also been developed to digest entire rats and mice permitting measurement of total body content of these trace elements.

2. Biological studies.

a. Neutron therapy of human disease.

Boron has a great physical capacity to absorb neutrons and concentrate the physical and tissue damaging effects of neutron irradiation. It is highly desirable to find ways to concentrate boron selectively in tumor tissues so that relatively high radiation dosage might be delivered to tumor tissues. The performance of meaningful studies in this area depends upon development of methods to measure tissue concentration of boron.

We have attempted to develop a sensitive procedure to measure boron concentrations in biological tissues. The method of Solway and Messer (Anal. Chem. 36, 433, 1964), has been modified to permit the safe use of 50% H_2O_2 as an oxidant after charring with fuming sulfuric acid. The reaction of iron in the ferrous state with 1,1-diathrimide produces a color that can be measured at 620 millimicrons. The method is sensitive to amounts of boron as low as 0.08 micrograms. This colorimetric measurement is linear up to boron quantities as great as 15 micrograms. The concentration of exogenously administered sodium borate appears to be relatively uniform in all mammalian tissues.

Polyvinyl alcohols are reported to stabilize borates in tissues. We have performed studies to determine borate fixing properties of polyvinyl alcohols which produce selective retention of borates in particular biological tissues. The results of our experiments are not encouraging; they seem to indicate that polyvinyl alcohols stabilize retention of borates in all tissue equally and are not likely to be useful in neutron therapy.

b. Tracer studies using cold stable nuclides.

Recently, investigators elsewhere have utilized enriched stable nuclides to study metabolism in newborn premature human infants. Highly enriched ^{58}Fe was administered orally to infants and blood samples were drawn thereafter. These samples were exposed to neutron flux in a reactor, and the induced radionuclide, ^{59}Fe, was measured. This method permits study of metabolism without exposure of the infant to irradiation and can be applied to pregnancy states in females as well. During the past several months, we have conducted pilot experiments to develop the techniques necessary to perform such studies with our nuclear reactor utilizing ^{58}Fe and ^{44}Ca. The latter nuclide may be useful in studies of calcium metabolism in pregnant women and infants.

c. Sodium-potassium content of dietary seafood and fish.

We have undertaken the following program in trace element analysis at the request of the Dietician, Food Service Division, Walter Reed General Hospital.

A number of fresh-caught seafoods are reported to be acceptable for use in diets of patients on restricted sodium and potassium intake. Most of these fish products as received by the hospital food service have been processed and/or frozen and are not fresh-caught. Commonly used chemical preservatives, such as propionates, and other additives commonly used in processing, contain sodium or potassium salts. Consequently, the relative dietary suitability of "fresh frozen" and "fresh processed" seafood and fish products is uncertain. In order to evaluate sodium and potassium content of these standard processed menu items, fresh frozen and processed seafood products will be chemically analyzed for total sodium and potassium content. Essential to this study is the development of

techniques for digesting and solubilizing tissue samples in a way that will not result in losses of sodium and potassium by volatilization of anion complexes of oils and fats. The samples received for study are lobster, shrimp, scallops, perch, salmon, sole,and halibut. This project is expected to be completed in three months or less.

d. Cystic fibrosis.

Studies elsewhere report increased sodium concentration in the fingernails of newborn human infants with cystic fibrosis. Activation analysis techniques are being developed here to reproduce these findings. We are now able to determine sodium concentrations reliably in 1-3 milligrams of nail tissues, and hope to offer this simple diagnostic test to pediatric services in all Army Medical Hospitals.

e. Iron metabolism in infection.

The role which the normal bacterial flora play in iron metabolism is poorly understood. A known complication of infection is anemia. This anemia is associated with alterations in iron metabolism which are characterized by hypoferremia, shortened plasma iron clearance, a slight increase in plasma iron transport rate, and a moderate decrease in red blood cell radioiron incorporation. Studies in germfree mice made possible examination of the effect of the germfree state on ferrokinetics.

We have performed studies of plasma radioiron clearance rate, 18-hour red blood cell radioiron incorporation, and tissue distribution of ^{59}Fe in germfree mice. In addition, the amount of iron in the spleen, liver, and plasma was determined by means of atomic absorption spectrometry. In a separate group of experiments total plasma iron concentrations were determined by standard chemical methods for comparison.

The results (Table I) demonstrated slight increase in the total plasma iron concentrations in the germfree compared to the conventionalized mice and excellent correlation between values obtained by the atomic absorption spectrometry technique. The splenic and hepatic iron concentrations were lower in the germfree mice.

Table II shows the tissue distribution of an injected tracer dose of radioiron in germfree and conventionalized mice. In the germfree animals, radioiron was more rapidly concentrated in the liver until 18 hours at which time there was no significant difference. The mean percent of the administered dose of radioiron in the spleen was greater in the conventionalized mice at all time intervals studied. The clearance of the radioiron from the plasma was markedly more rapid in the germfree mouse and the plasma iron transport rate was increased indicating more rapid plasma turnover. The 18-hour red blood cell radioiron utilization, however, was not significantly different.

TABLE I

HEMATOCRIT, ORGAN WEIGHTS AND TISSUE IRON CONTENT

	Germfree	Conventionalized	Significance[3]
Body weight gm ± S.D.[1]	31.0 ± 2.9 (40)[2]	31.7 ± 1.7 (40)	N.S.[4]
Liver weight gm ± S.D.	0.855± 0.149 (34)	1.274± 0.240 (32)	P < 0.05
Spleen weight ug ± S.D.	79.4 ± 17.3 (32)	98.9 ± 17.1 (32)	P < 0.05
Hematocrit vol % ± S.D.	45.1 ± 0.3 (32)	42.4 ± 3.6 (32)	N.S.
Plasma Iron[5] ug/ml ± S.D.	7.82 ± 2.42 (32)	6.04 ± 1.12 (32)	P < 0.05
Liver Iron[5] ug/gm fresh liver ± S.D.	279.3 ± 70.6 (32)	308.3 ± 60.4 (32)	P < 0.05
Spleen Iron[5] ug/gm fresh spleen ± S.D.	941.1 ±417.3	1364.4 ±435.8	P < 0.05

[1]Standard Deviation: S.D.

[2]Number of mice in parentheses.

[3]Determined by Student's "t" test.

[4]Not significant: N.S.: probability > 0.05.

[5]Determined by atomic absorption spectrometry.

TABLE II

TISSUE DISTRIBUTION OF RADIOIRON

	Time Following I.V. ^{59}Fe Administration			
	30 Min	60 Min	90 Min	18 Hours
Liver % ± S.D.[1]				
Germfree	4.8 ± 1.6(8)[2]	4.9 ± 1.8(8)	5.2 ± 1.3(8)	12.5 ± 3.0(12)
Conventionalized	6.1 ± 1.8(8)	7.5 ± 1.7(8)	8.7 ± 1.4(8)	13.9 ± 1.9(12)
Spleen % ± S.D.				
Germfree	1.3 ± 0.6(8)	1.3 ± 0.7(8)	2.5 ± 1.1(8)	2.3 ± 1.2(12)
Conventionalized	2.0 ± 1.0(8)	4.3 ± 2.5(8)	3.8 ± 2.2(8)	5.8 ± 2.1(12)
Plasma % ± S.D.				
Germfree	70.4 ± 18.2(8)	50.7 ± 11.0(8)	35.6 ± 4.0(8)	---
Conventionalized	84.0 ± 16.0(8)	76.9 ± 14.3(8)	65.7 ± 12.9(8)	---
Red Blood Cell % ± S.D.				
Germfree	---	---	---	27.2 ± 5.6(12)
Conventionalized	---	---	---	33.1 ± 6.9(12)

[1]Standard Deviation: S.D.

[2]Number of Mice in Parentheses.

These results indicate that bacterial contamination with nonpathogenic organisms is of importance in controlling ferrokinetics and erythropoiesis.

f. Trace metals in wound healing.

Iron, copper, and zinc are essential in man. These elements lie within the transition zone between micronutrient and macronutrient substances. The metabolism of these trace elements has been extensively investigated clinically in health and disease; however, it was not until recently that biological tissue concentrations of these semi-micronutrients have been studied in particular clinical situations. An acceleration of the rate of wound repair has followed the exogenous administration of zinc in man and experimental animals, suggesting that zinc may be involved with wound healing. However, the concentration of trace metals in the healing wound tissue has not been adequately correlated with wound repair. Therefore, we have measured the iron, copper, and zinc concentrations in the granulation tissue bed sequentially following wounding. A standard open skin wound was produced in rats and serially sampled (see WRAIR Annual Report, 1967). The rate of contracture was subsequently determined and animals were sacrificed 6 hours, 1, 2, 3, 4, 8, and 12 days following wounding and the iron, copper, and zinc content of the granulation tissue was determined by means of atomic absorption spectrometry.

The concentration of iron, copper, and zinc was measured in the granulation tissue bed of wounds in normal animals and animals previously irradiated (prior irradiation has been shown to produce a delay in wound repair). Rats were wounded four days following exposure to 675 R X-ray and sacrificed at times following wounding as above.

Tissue iron concentration decreased until the second day following wounding. The level of iron subsequently increased up until the eighth day and then diminished toward the initial level by the twelfth day. The iron pattern in the irradiated wound shows an initially higher iron content with a delay in the decrease, reaching the nadir by the fourth day, and thence increasing in concentration through the twelfth day. The pattern seems to reflect approximately a four-day delay in change in iron levels in the irradiated wound. This observation is consistent with the observed delay in wound contraction following irradiation (see Basic Research in Support of Military Medicine: Radiobiology, Mechanisms, WRAIR Annual Report 1968).

The copper content of the nonirradiated wound remains remarkably constant during the 12 day period studied. The copper content of the irradiated wound, although elevated six hours following wounding and again slightly elevated on the third day, was essentially the same as the nonirradiated wound during the remainder of the 12-day study. The slight increase noted on the third day in the irradiated wound could not be correlated with any other event in wound healing. However, the initial six hour increase in copper correlates with the initial pronounced increase in wound area in the irradiated animal.

In the nonirradiated animals the zinc pattern was characterized by a decrease between six and 24 hours, relative stability between Days 1 and 4, then a subsequent steady increase up to and including Day 12. The rate of wound contracture was rapid for the first four days, and less rapid rate to Day 12, which was the period of augmented zinc concentration.

Further studies relating these changes in trace metals with the total metabolic pattern are presently underway.

3. Clinical studies.

In order to further delineate the normal range of copper, zinc, iron, and manganese levels in man, nine apparently healthy male subjects ranging in age from 20 to 45 years were selected. Plasma samples were taken by venipuncture twice in a 60 day interval; urine was collected on four successive days; and samples of fingernails and hair were also examined. Copper, iron,and zinc levels in the various samples were measured by means of atomic absorption spectrometry, while manganese was determined by neutron activation analysis. In order to determine the variability of ordinary urinary constituents, analyses were also performed for urea-N, creatinine and urinary potassium excretion in the 24-hour urines. Correlation coefficients for the creatinine excretion in relation to body weight were calculated as an index of the adequacy of the urine collection.

The results of this study are tabulated in Tables III, IV, and V.

TABLE III

PLASMA LEVELS OF COPPER, MANGANESE, IRON AND ZINC

Variable	Mean ± SD micrograms/ml		Range micrograms/ml
Copper	10/67	1.33 ± 0.118	1.1 - 1.4
	1/68	1.31 ± 0.095	0.99 - 1.26
Manganese	10/67	0.0495 ± 0.0353	0.020 - 0.117
	1/68	0.0837 ± 0.0536	0.013 - 0.159
Iron	10/67	1.26 ± 0.326	0.70 - 1.80
	1/68	1.33 ± 0.352	1.03 - 1.80
Zinc	10/67	1.55 ± 0.316	1.11 - 1.90
	1/68	1.35 ± 0.510	0.78 - 2.02

TABLE IV

URINARY EXCRETION OF COPPER, MANGANESE, IRON, ZINC, CREATININE, UREA-N, POTASSIUM AND TOTAL SULFUR OVER FOUR SUCCESSIVE DAYS

Variable		Mean ± Standard Deviation	Range
Urea-N gms/day	1	13.1 ± 3.193	8.5 - 17.2
	2	12.1 ± 3.159	8.0 - 17.0
	3	13.0 ± 3.390	8.5 - 18.1
	4	11.6 ± 3.059	5.4 - 15.9
Total Sulfur gms/day	1	1.28 ± 0.380	0.6 - 1.7
	2	1.33 ± 0.335	0.9 - 1.9
	3	1.49 ± 0.419	0.9 - 2.1
	4	1.40 ± 0.287	1.0 - 1.9
Potassium gms/day	1	2.81 ± 0.637	1.8 - 4.0
	2	2.85 ± 0.894	1.7 - 4.3
	3	2.96 ± 0.328	1.5 - 4.7
	4	3.11 ± 2.478	1.2 - 8.8
Copper microgms/day	1	86.2 ± 33.97	44 - 145
	2	75.7 ± 43.62	26 - 128
	3	73.6 ± 22.29	40 - 102
	4	76.5 ± 52.91	36 - 205
Manganese microgms/day	1	1.93 ± 0.860	0.97 - 3.7
	2	3.97 ± 3.633	0.80 - 10.6
	3	2.11 ± 1.486	0.75 - 3.8
	4	1.01 ± 0.645	0.30 - 2.2
Iron microgms/day	1	88.78 ± 18.35	59 - 117
	2	56.00 ± 12.85	38 - 78
	3	91.33 ± 63.90	38 - 240
	4	66.67 ± 20.22	41 - 99
Zinc microgms/day	1	539 ± 114	349 - 678
	2	479 ± 42.5	336 - 714
	3	553 ± 182	368 - 980
	4	520 ± 189	237 - 822

Creatinine versus Body weight	Day 1	2	3	4
Correlation Coefficient	0.55	0.65	0.60	0.52

TABLE V

ANALYSIS OF FINGERNAILS AND HAIR FOR COPPER, MANGANESE,

IRON AND ZINC IN NORMAL SUBJECTS

Fingernails	Mean micrograms/gm	Range micrograms/gm
Copper	23.5	8 - 42
Manganese	1.15	0.31 - 3.11
Iron	230.8	106 - 450
Zinc	161.4	94 - 260
Hair		
Copper	25.7	10 - 39
Manganese	0.34	0.23 - 0.46
Iron	118.5	60 - 192
Zinc	137.2	106 - 215

TABLE VI

INTERCOMPARISON OF NORMAL TRACE ELEMENT LEVELS IN PLASMA AND URINE IN NORMALS AND PATIENTS WITH CIRRHOSIS, ACHALASIA AND ULCERATIVE COLITIS

	Copper	Manganese	Iron	Zinc
Normal				
Plasma microgms/ml	1.32	0.0666	1.295	1.45
Urine microgms/day	78.0	2.25	75.69	522
Cirrhosis				
Plasma microgms/ml	1.36	0.017	1.92	0.845
Urine microgms/day	70.5	4.0	118	520
Achalasia				
Plasma microgms/ml	1.37	0.014	1.08	1.20
Urine microgms/day	70.5	4.6	62.0	650
Ulcerative Colitis				
Plasma microgms/ml	1.54	0.005	0.92	0.92
Urine microgms/day	61.0	13.2	95.0	580

Plasma copper, iron, and zinc levels were reproducible within the two sampling periods. Manganese levels showed the greater fluctuation in mean values as well as the greatest excursion in range.

Urinary excretion levels of all the variables measured showed a great deal of day-to-day variability. Attempts were made to correlate the variables with age and urinary volume with no success. Therefore, it should be pointed out that plasma levels mirror the status of the major transport fluid at the time of sampling and that urinary output apparently reflects the action of the kidney.

Intercomparisons were made between normals and groups of patients with clinically defined cirrhosis, achalasia, and ulcerative colitis. The results are presented in Table VI.

Copper levels in plasma and urine in the three groups studied were remarkably similar to normal. In all three clinical disorders, manganese levels in plasma were reduced while urinary excretion was elevated, with the highest elevation occurring in the ulcerative colitis patients. Plasma iron and urinary iron excretion was elevated in cirrhosis and near normal in the other two groups. Plasma zinc levels and urinary excretion appeared within normal ranges in the patients studied.

The continued collection of data on plasma levels and urinary excretion of trace elements in clinically defined disease states is required to define more clearly the meaning of such data. In addition, analyses of other body fluids may provide additional information on the influence of trace metals in gastrointestinal disease.

Summary and Conclusions.

During the past year, we have improved our methods in measurement of trace metal concentrations in biological systems. Quantities of iron, copper, zinc, and magnesium that are usually found in biological samples can be measured to plus or minus three percent.

These methods have been applied to several clinical situations:

1. Neutron activation analysis of sodium in fingernail clippings is being evaluated as a screening test for cystic fibrosis in newborns.

2. Methods are being developed to utilize enriched nonradioactive nuclides, ^{58}Fe and ^{44}Ca, in performance of metabolic studies in newborns and pregnant females.

3. At the request of the WRGH Dietician, the sodium and potassium content of certain seafoods is being measured to evaluate their suitability for restricted diets.

4. Plasma levels and urinary excretion rate of copper, zinc, iron,

and manganese have been determined in nine apparently normal human males. Considerable variation was found.

5. Plasma levels and urinary excretion rates of copper, zinc, iron, and manganese have been determined in approximately 30 patients with either cirrhosis, achalasia of the esophagus, or ulcerative colitis. Minor variations from normal have been found.

Studies have been performed in germfree and conventionalized mice to investigate the effect of normal bacterial contamination on iron metabolism. Plasma iron clearance is more rapid in germfree mice, and organ iron content is also significantly different in these two states.

The role which trace metals play in wound healing has been investigated in irradiated and normal rats. The iron content of granulation tissue is increased in the wounds of animals which have been irradiated and this change occurs during the interval of greatest radiation-induced delay in wound healing.

Publications.

1. Cheek, D. B., R. C. Reba and K. T. Woodward. Chapter 30, Cell Growth and the Possible Role of Trace Minerals (Cu, Zn, Mn) in "Human Growth", pp. 424-439, ed. Donald B. Cheek, Johns Hopkins Univ., publ. Lea & Febiger, Philadelphia, Penna., in press (1968).

2. Reba, R. C., D. B. Cheek and F. C. Leitnaker. Chapter 11, Body Potassium and Lean Body Mass in "Human Growth", pp. 165-181, ed. Donald B. Cheek, John Hopkins Univ., pub. Lea & Febiger, Philadelphia, Penna., in press (1968).

3. Reba, R. C., F. C. Leitnaker and K. T. Woodward. Chapter 45, Part 2, Determination of Total Body Potassium by Measurement of 40 K in "Human Growth", pp. 674-681, ed. Donald B. Cheek, Johns Hopkins Univ., pub. Lea & Febiger, Philadelphia, Penna., in press (1968).

4. Smathers, J. B., D. Duffey and S. Lakshmanan. "Survey of Chromatographic Papers for Interfering Nuclides in Activation Analysis", Anal. Chim. Acta 39:529-532 (1967).

5. Smathers, J. B., S. Lakshmanan and D. Duffey. "Derivative Activation Analysis of Magnesium", Trans. Amer. Nucl. Soc. 10:448 (1967).

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CA0530	CSGRD-103

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

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 171

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) AOTUS TRIVIRGATUS AS A LABORATORY ANIMAL

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
CC2600 BIOLOGY	07 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
B. CONTRACT	b. NUMBER DADA-17-67-C-7176 07 67	PRIOR FY 68	1	180
	c. TYPE A.FPF d. AMOUNT $358,772	CURRENT FY 69	2	178

19. GOVT. LAB. INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
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TEL: 202-576-3551	ASSOCIATE: TIGERTT, COL W. D.
	TEL: 202-576-3551 TYPE: DA

21. TECHNOLOGY UTILIZATION	22. COORDINATION
MEDICINE	NA

23. KEYWORDS: PRIMATE, MALARIA, MICROBIOLOGY.

24.

(U) TECH OBJECTIVE - TO ESTABLISH ACTUS TRIVIRGATUS (NIGHT MONKEY) AS A LABORATORY PRIMATE AND TO CHARACTERIZE INFECTIONS WITH PLASMODIUM FALCIPARUM IN THIS HOST.

(U) APPROACH- ESTABLISH REQUIREMENTS FOR MAINTAINING HEALTHY ANIMALS, INFECT BY SPOROZOITE AND BY BLOOD INJECTION, DEVELOPE METHODS OF ASSESSMENT OF ANTIMALARIAL DRUGS.

(U) PROGRESS - JUL 67 THRU JUN 68 NO SIGNIFICANT HUSBANDRY PROBLEMS HAVE BEEN ENCOUNTERED. INTACT ANIMALS CAN BE INFECTED WITH PLASMODIUM FALCIPARUM BY INTRAVENOUS OR INTRAPERITONEAL ROUTES. WITH APPROPRIATE INITIAL DOSES, PARASITEMIA IS PREDICTABLE AND ABOUT 90 PERCENT OF THE INFECTIONS TERMINATE FATALLY. INITIAL STUDIES INDICATE THAT 4-AMINOQUINOLINES PRODUCE A RESPONSE IN AOTUS COMPARABLE TO THAT SEEN IN MAN ON A WEIGHT BASIS. OTHER STANDARD ANTIMALARIALS ARE BEING EXAMINED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY-INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 171, Aotus trivirgatus as a laboratory animal

Investigators.
Principal: L. H. Schmidt, Ph.D.
Associate: R. N. Rossan, Ph.D.

Description.

The immediate objective of this investigation was to stabilize and standardize infections evoked by two different strains of Plasmodium falciparum in the Aotus monkey. As this gave promise of accomplishment, a new objective was incorporated which was to appraise the response of developed infections with these strains to chloroquine and pyrimethamine. A very limited and low priority objective was to determine the disease-producing qualities of a Vietnam strain of P. vivax in Aotus.

Progress.

Serial transfer of both the Malaysian Camp. and Uganda-Palo Alto strains of P. falciparum through the intact Aotus have brought these lines to a place where they produce almost uniformly fatal infections with inocula of 10^4 parasites or more. With an inoculum in the 10^6 to 5 X 10^6 range, deaths occur 9 to 18 days after inoculation. In such cases parasitemias range from 3000 to over 10,000 per 10^4 erythrocytes. Multiple infections of red cells are extremely common, accounting for the fact that at times parasite numbers do exceed numbers of erythrocytes. An attempt is now in progress to determine whether there is a polymorphism in the different subjects which might be associated with their susceptibility to falciparum infections.

In general, results obtained thus far do not indicate any distinct differences in the pathogenicity of the strains. All fatalities occurred with parasitemias in excess of 30% and the mortality rate itself fluctuates between 30% and 36% for the two strains of falciparum. Other results show that (1) an inoculum of about 10^3 parasites is required to produce infection; (2) the thick film demonstrable "pre-patent period" varies inversely with inoculum size; (3) the "pre-patent period" is 1 to 3 days shorter with IV than with IP inoculation; and (4) once parasitemia is established the course of the disease is independent of inoculum size or route of infection.

With the exception of the first shipment, losses of Aotus from causes other than malaria have been negligible. In the first group of 50, however, 45 died within 14 days of delivery with symptoms of acute upper respiratory infection. The cause of death has been shown to be Herpes simplex.

Systematic assessments have been made of the susceptibilities of both falciparum strains to chloroquine. Two experiments have been performed with each strain. Groups of 20 monkeys were inoculated with 2×10^6 trophozoites. When parasitemias approximated 10^2 per 10^7 red cells, subgroups of 5 monkeys were assigned as untreated controls or were treated with 1.25, 5.0, or 20.0 mg base/kg body weight, daily for 7 days. Surprisingly, the response of the supposedly chloroquine-susceptible Uganda-Palo Alto strain and the supposedly chloroquine-resistant Malaysian Camp. strain have been essentially the same. Doses of 1.25 mg/kg checked parasite development, but did not promote parasite clearance. Doses of 20.0 mg/kg effected trophozoite clearance in 2-3 days as compared with 3 to 5 days for doses of 5.0 mg/kg. On the basis of all previous experience the conclusion to be drawn from this is that both strains are susceptible to chloroquine. It must now be determined insofar as the Camp. strain is concerned (1) whether a return to susceptibility has occurred during the unlimited multiplication in Aotus with a resultant breeding out of a susceptible mutant, or (2) whether contamination with a susceptible strain has occurred in one or another of the institutions in which this strain was maintained previously.

Work is proceeding on evaluations of the susceptibility of infections with the Uganda-Palo Alto strain to other antimalarials. A single study on pyrimethamine susceptibility has been completed. Results indicate that doses of 0.6 mg base per kg body weight daily for 7 days fail to control parasitemia. Based on experiences with other plasmodia, the Uganda-Palo Alto strain would have to be considered pyrimethamine resistant.

Summary and Conclusions.

a. Experience with several hundred specimens shows that a reasonably well stabilized and standardized infection with Plasmodium falciparum can be induced in the intact Aotus trivirgatus monkey without particular difficulty.

b. All passages thus far have been made with blood.

c. On the basis of current experience it should be possible to pinpoint a new potential drug's activity against P. falciparum by using no more than three monkeys and possibly as few as one.

d. No differences have been seen between the Malaysian Camp. and Uganda-Palo Alto strains of P. falciparum used in this study.

e. Limited observations suggest that the supposed chloroquine-resistant Camp. strain is as susceptible to chloroquine as the Uganda-Palo Alto strain. This is thought to be attributable either to a laboratory accident in which strains became confused or that the rapid multiplication of the parasite in Aotus has permitted the emergence of a chloroquine-sensitive mutant.

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 086539	CSCRD-100

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

10a. CURRENT NUMBER/CODE: 61103011 3A013001A91C 00 172

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) MIGRATORY ANIMAL PATHOLOGICAL SURVEY

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	07 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT		18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
C. IN-HOUSE	a. NUMBER NA	b. DATE NA	PRIOR FY 68	4	59
	c. TYPE NA	d. AMOUNT NA	CURRENT FY 69	3	60

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21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NA

23. KEYWORDS: ORNITHOLOGY, MIGRATION, VECTORS.

24.

(U) TECH OBJECTIVE - PARTICULAR INTEREST IS IN THE ROLE OF MIGRATING ANIMALS IN THE TRANSPORT OF DISEASE.

(U) APPROACH- MAJOR EFFORT WILL BE ON BIRD BANDING AND RECOVERY IN VARIOUS AREAS OF SEA. ECTOPARASITES WILL BE COLLECTED, BLOOD AND TISSUES WILL BE EXAMINED. AREA SUPERVISION WILL BE FROM BANGKOK.

25 (U) PROGRESS - JUL 67 THRU JUN 68 STATIONS HAVE BEEN ESTABLISHED IN TAIWAN (DA 089503), KOREA (DA 089509), PHILIPPINES (DA 089510, DA 088513), MALAYSIA (DA 089511), JAPAN (DA 089512), THAILAND (DA 089514, DA 086515), SARAWAK (DA 089516), SABAH (DA 089518), INDIA (DA 089538), INDONESIA (DA 089525). DETAILED REPORTS TO BE SUBMITTED BY CONTRACTORS THROUGH ARO(FE).

26.

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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1127)

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 086420	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	C. CHANGE 05 06 67	U U	NA	GA	A. WORK UNIT

10a. CURRENT PROGRAM CODE: 61130011 3A013001A91C 00 173

10b. PRIOR NUMBER CODE:

11. TITLE: (U) THERMAL PROPERTIES OF LIQUID WATER

12. SCIENTIFIC OR TECH. AREA: 012700 THERMODYNAMICS

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
06 67	NA	OTHER DA

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

16. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
FY 68	1	32
FY 69	1	20

18. GOVT. LAB/INSTALLATION/ACTIVITY
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ASSOCIATE:
TEL: 202-OX4-3346 TYPE: DA

21. TECHNOLOGY UTILIZATION: PHYSIOLOGY
PHARMACOLOGY

22. COORDINATION: NA

23. KEYWORDS: WATER STRUCTURE, WATER ANOMALIES, TEMPERATURE DEPENDENCE OF PHYSICAL PROPERTIES, DIFFERENTIAL CALORIMETRY, VAPOR-PRESSURE OSMOMETRY.

24.

(U) TECH OBJECTIVE - TO ELUCIDATE THE TEMPERATURE DEPENDENCE OF THE PHYSICAL PROPERTIES OF LIQUID WATER AND THEIR RELATIONSHIP TO LIVING SYSTEMS.

(U) APPROACH- IT HAS BEEN HYPOTHESIZED THAT WATER STRUCTURE CHANGES DISCRETELY INTO SPECIFIC STRUCTURAL FORMS AT VARIOUS TEMPERATURES AND THAT THE STRUCTURES DOMINATING THE VARIOUS RANGES CONSTRAIN LIVING PROCESSES BECAUSE OF THE LONG ASSOCIATION DURING EVOLUTION OF THE MACROMOLECULES AND MEMBRANES WITH STRUCTURED WATER. THIS MAY BE THE REASON WHY LIVING ORGANISMS SHOW TEMPERATURE OPTIMA AND MINIMA IN GROWTH AND FUNCTION. RECENTLY BACH AND REZER HAVE DETERMINED BY DIFFERENTIAL CALORIMETRY OF PURE WATER THAT THE RECENT THERMAL HISTORY OF WATER DETERMINES ITS THERMAL PROPERTIES, THAT IS WATER WHICH HAS RECENTLY BEEN HEATED IS PHYSICALLY DIFFERENT FROM WATER WHICH HAS NOT, EVEN THOUGH BOTH SAMPLES ARE AT THE SAME TEMPERATURE.

(U) PROGRESS - JUN 67 THRU JUN 68 THE EXOTHERMS OBSERVED WHEN A PREVIOUSLY HEATED SAMPLE OF WATER IS COMPARED BY DIFFERENTIAL CALORIMETRY WITH ONE WHICH HAS NOT, HAVE BEEN SHOWN TO BE DUE TO A FILM OF WATER WHICH DEPOSITS ON THE WALL OF THE SAMPLE WELL DURING COOLING OF THE SAMPLE. THIS FILM DEPOSITS IN SEVERAL LAYERS EACH CHARACTERISTIC OF THE TEMPERATURE RANGE IN WHICH IT IS FORMED. IT EVAPORATES IN DISTINCT STEPS DURING REWARMING, REPETITIVELY SUPPRESSING EVAPORATION IN THE SAMPLE CUP, WHICH THEREFORE WARMS FASTER THAN THE REFERENCE DURING EACH STEP PRODUCING EXOTHERMIC PEAKS. THESE LAYERS ARE HIGHLY PERSISTENT. THE VAPOR PRESSURE OF THE HIGH TEMPERATURE FORMS IS INITIALLY LOWER THAN THAT OF THE BULK WATER IN THE CUP, AND THEREFORE THEY RESIST EVAPORATION, EVEN THOUGH WARMER THAN THE SAMPLE, UNTIL THE SAMPLE ATTAINS A STATE HAVING AN EQUAL OR LOWER VAPOR PRESSURE. THESE TEMPERATURE DEPENDENT FORMS MAY BE THE BASIS FOR THE NUMEROUS OBSERVATIONS OF ABRUPT, TEMPERATURE DEPENDENT ALTERATIONS IN PHYSICAL AND BIOLOGICAL PROPERTIES OF AQUEOUS SYSTEMS AS REVIEWED AND REPORTED BY DROST-HANSEN. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In thousands)	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A0130C1A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 173, Thermal properties of liquid water.

Investigator.

Principal: Sven A. Bach, M.D.

Description.

Evidence is mounting that water may exist in discrete forms in various temperature ranges. If this is so, the structure of water may have profoundly influenced the form and function of enzymes and other macromolecules, as well as the structure and function of biological membranes. Elucidation of these structures and the application of this knowledge to military problems, is the object of this research.

Progress.

Inasmuch as this project will be terminated 30 Sep 68, the investigations conducted between 1 Jul 67 and 30 Jun 68 will be incorporated in the final report on the project.

Summary and Conclusions.

None

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 046421	CSCSD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY (a. RDT, b. WHO)	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
31 07 68	D. CHANGE 01 06 67	a. U b. U	NA	6A	A. WORK UNIT

10a. CURRENT NUMBER/CODE
6113061I 3A013001A91C 00 174

10b. PRIOR NUMBER/CODE

11. TITLE (U) APPLICATIONS OF ELECTROANALYTICAL TECHNIQUES TO BIOCHEMISTRY AND CLINICAL CHEMISTRY

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
CC2300 BIOCHEMISTRY	06 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
D. CONTRACT	a. NUMBER DADA17-67-C-7161 b. TYPE A.FPF c. AMOUNT $53,226	PRIOR FY 68	1	26
		CURRENT FY 69	1	27

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21. TECHNOLOGY UTILIZATION: BIOCHEMISTRY; TOXICOLOGY

22. COORDINATION: NA

23. KEYWORDS
CHEMISTRY, CLINICAL, ANALYTICAL, ELECTROCHEMICAL, ELECTROLYTES, BIOCHEMISTRY.

24.

(U) TECH OBJECTIVE - TO EXTEND THE PRECISION OF ELECTROANALYTICAL CHEMISTRY TECHNIQUES TO CLINICAL CHEMISTRY ANALYSES AND TO INVESTIGATIVE BIOCHEMISTRY, TO PROVIDE PRIMARY REFERENCE METHODS FOR EVALUATION OF CLINICAL AND INVESTIGATIVE CHEMISTRY METHODOLOGY.

(U) APPROACH- ELECTROANALYTICAL PROCEDURES, INCLUDING COULOMETRY, POLAROGRAPHY, CHRONOPOTENTIOMETRY AND ASSOCIATED TECHNIQUES, WILL BE APPLIED TO ANALYSES OF BIOCHEMICAL COMPOUNDS. ALL DETAILS OF THE REACTIONS WILL BE STUDIED IN ORDER TO DEVELOP HIGHLY PRECISE AND SPECIFIC ANALYSES WHICH WILL BE AVAILABLE AS PRIMARY REFERENCE METHODS AGAINST WHICH OTHER METHODS AND PROCEDURES CAN BE COMPARED.

(U) PROGRESS - JUN 67 THRU JUN 68 A TITRATION METHOD FOR DETERMINATION OF URIC ACID IS BASED ON OXIDATION WITH COULOMETRICALLY GENERATED IODINE. OXIDATION OF URIC ACID AT PH VALUES GREATER THAN 8 INVOLVES A 2 ELECTRON CHANGE TO ALLOXANIC ACID. FAST-SWEEP POLAROGRAPHY INDICATES AN ALLOXAN IS AN INTERMEDIATE IN THIS OXIDATION. A URICASE DIFFERENTIAL METHOD TO CONVERT URIC ACID TO ALLANTOIN AND HYDROGEN PEROXIDE IS NEEDED. ALLANTOIN DOES NOT REACT WITH IODINE, HYDROGEN PEROXIDE IS REMOVED BY INCORPORATING CATALASE IN THE URICASE PREPARATION. CHILLING SOLUTIONS REDUCES SECONDARY REACTIONS AND DOES NOT AFFECT TITRATION OF URIC ACID WITH IODINE. COULOMETRIC RESULTS ON 250 MICROLITERS OF SERUM AGREE WITH VALUES FROM SPECTROPHOTOMETRIC METHODS. DIFFERENTIAL DERIVATIVES CHRONOPOTENTIOMETRY IS BEING COMPARED WITH AUTOMATIC CURRENT REVERSAL DATA TO CHECK EFFICIENCY CIRCUIT DESIGN. THE RATIO OF FORWARD TO REVERSE TIMES TO INITIAL FORWARD T (TAU) FOLLOWS DEFINITE NUMERICAL ORDERS FOR VARIOUS SYSTEMS. THESE TWO TECHNIQUES ARE BEING USED TO STUDY THE ROLE OF METAL IONS IN ENZYMATIC REACTIONS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
a. COVERS OR COVERS RELATED ☐ b. NOT RELATED ☒		6R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In Thousands)	36.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 174, Applications of electroanalytical techniques to biochemistry and clinical chemistry

Investigators.
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COL E. C. Knoblock, MSC
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Description.

This program was designed to investigate the potential applications of the precise analytical techniques of electroanalytical chemistry with the expectation that highly accurate and specific processes could be developed for the characterization and assay of biochemical substances. In addition to providing potential reference methods for analytical procedures, the chemical reactions of biochemical species in varying chemical environments were to be investigated.

Progress.

Four general techniques have been employed during this study, atomic absorption, chronopotentiometry, coulometry, and polarography.

1. Atomic absorption. Preliminary studies have been instituted for the application of atomic absorption spectroscopy to the determination of trace metals in biological systems. The determination of vanadium is of interest because vanadium has the requisite atomic structure for a catalyst and it is known to oxidize a number of biologically active compounds. It has many biological activities *in vivo* and *in vitro*, and has been associated with lipid metabolism by Schroeder *et al*.

Vanadium levels in whole blood have been found to be elevated in various types of anemia and in leukemia.

Most determinations of vanadium in biological materials have used spectrographic or spectrophotometric methods. The nitrous oxide-acetylene flame developed by Willis has made it possible to determine vanadium by atomic

absorption with a sensitivity of 1.5 μg/ml. Jaworowski et al. have reported a sensitivity enhancement of two orders of magnitude in aqueous solution, and 2.4 orders of magnitude in 80% methanol, when a nitrous oxide-acetylene flame replaced the standard air-acetylene flame.

Extraction into an organic solvent is a generally useful technique in atomic absorption. Crump-Wiesner and Sachdev et al. have determined vanadium by atomic absorption with a nitrous oxide-acetylene flame, using extraction with cupferron into methyl isobutyl ketone (MIBK). Of the digestion procedures for use in the determination of vanadium in biological materials, wet ashing is preferable because dry ashing in porcelain can introduce significant amounts of vanadium into the sample.

2. Chronopotentiometry. Most of the work in this area was concerned with recognizing and working out "bugs" in the electronic circuitry. Some calibration data have been obtained and sufficient chronopotentiometric runs obtained with 1 mM cadmium ion to ascertain the correct function of the circuit with its changes.

The relay control of the control loop is superior to diode control, as the diodes give non-linear response. Some trouble was encountered in the switching amplifier. It was found that the best way to drive the relay was by taking a signal from the comparison amplifier, through a positive feedback switching amplifier, to the relay, and on to the 15-volt power supply. An input switch permits the relay to be disabled. Relay drive to the -15 volt supply permits removal of a diode and gives higher voltage to operate the relay, resulting in more rapid and positive relay action. Attempts to operate the switching amplifier and relay from a second derivative of the E-volume failed.

Attempts to reverse the chronopotentiometric run at τ proved very difficult with a manual switch. A second pole of the control loop relay was used to give automatic control to Forward and Reverse relays that paralleled the manual switch.

With added amplifiers, it was necessary to use three separate power supplies and undesirable grounding problems arose. A Philbrick PR 300 solid state power supply was obtained and an extension was added to the chassis to contain five more amplifiers, three relays, and other switches. The entire unit is now powered from the one power supply and functions well.

The Forward input relay is energized through one of its own normal open contacts, which are paralleled with a momentary push button to begin a chronopotentiometric run. The Forward and Reverse relays are activated by the control loop relay as these take too much current to be driven from an amplifier directly. Another switch further provides the following modes of operation:

(1) Forward mode until τ, then stop;

(2) Forward mode until τ, Reverse mode until τ', then stop; and

(3) Forward mode until τ, Reverse mode until τ', - continuing cycle.

These Forward-Reverse relays have extra poles available to permit the operation of timers to record the τ and τ' values directly. In trial runs, essentially the same times are determined by evaluating the E-t curve, the dE/dt-t curve, or relay controlled timer. Apparently τ, the drop in potential to zero at the comparison amplifier output and the relay action all occur within a few milliseconds.

It is best to open the switching amplifier input before pressing the Forward push button to ensure an inactive control loop relay and its normal closed points feeding current to the Forward relay as soon as the push button is released. In an actual run, it is only necessary to remember to put the switching amplifier and control relay in the circuit before τ is reached; they are needed to prevent positive feedback. To avoid some rapid relay oscillations, it was best to use a positive feedback switching amplifier which has about 3 mV hysteresis lag on its input signal.

There is also a problem of noise that is picked up at the Test SCE and carried all the way to the dE/dt or log outputs. Attempts at inserting a crude 60 cycle R+C rejection filter did not warrant continued use. A 100 K feedback resistor added to parallel the diodes in the log amplifier decreases the sensitivity in the 0.1 mV range and gives essentially ± 5 decades on the recorder. This lower sensitivity to noise helps, as did combining all amplifiers in the one chassis and under one power supply. Some problem with recorder chatter and noise arises on log or derivative readout that is not directly associated with 60-cycle noise as tested with a signal generator. Further noise elimination may be needed.

To test the comparison mode, two integrators were built around chopper stabilized amplifiers run from floating power supplies and low leakage capacitors. They were calibrated as follows:

Q3 amplifier with 1 meg/1 μF gave 0.1492 volt/sec = 2.010 mV/coulomb

UPA-2A with 1 meg/0.1 μF gave 1.491 volts/sec = 0.2011 mV/coulomb

The voltage drops across matched precision resistors in the Blank anode and Test input circuits would not give the same integration values. This showed that compensation currents were not equal to the blank current, which condition, it is assumed, should be met. An error varying with the output of the Blank cell was caused by a current through the 1 K potentiometer input to the scaling amplifier and a resistor network to ground. This was avoided by connecting this network before the 1 K potentiometer, at the expense of reversing the output polarity of the scaling amplifier and necessitating using another amplifier to invert the signal appropriate to apply to the Test cell.

Since none of the eleven amplifiers are stabilized, careful attention to balancing each one is needed. With no input, and in the Reverse-compensation mode, it is found that the two SCE's do not match and about +22 mV bias potential is needed to bring the Blank SCE equal to the Test SCE. This difference is attributed to SCE and cell parameter differences. A bias control should be added to adjust for this difference.

Attempts to make a digital readout integrator, patterned after some in the literature, did not give the desired accuracy. Long time (5-10 min) values could be obtained nicely, but 20-40 sec intervals would give too few counts for needed precision.

Recently, problems with oscillations occurring while in the compensation mode and near τ gave reason to again consider using counter electrodes other than platinum. Lead-in wire connections were made to the Pt wires in a small pool of mercury covered with black wax. The 10-mm tubes were packed with mercury, calomel, and cellulose powder moistened with saturated KCl. They were closed with a plug of cotton also wet with KCl, and the whole assembly inserted into a glass-frit tube filled with KCl. This electrode reads about -0.15 V to the mercury pool as does the indicating micro SCE. Initial runs with these counter electrodes looked promising, but it was soon found that passing high currents through them caused poor results. A set of current-potential data were obtained to study the range over which the SCE was unpolarized, and this proved to be about +0.1 to -1.0 volts vs. the Hg pool. To pass appreciable currents (over 20 μA), the potential must be outside this range. Once the electrodes were polarized, they did not return very rapidly to their previous values. As a result, in the compensation cell set-up, they supplied a compensating current in the forward direction, when the test cell called for the current in the reverse direction, and resulted in runaway amplifiers. These unexpected results brought to light the importance of polarization effects at both electrodes and a brief study was undertaken after returning to platinum counter electrodes.

Several circuit changes were made as follows:

a. Attempts at using silicon-controlled rectifiers and transistors as switching units in place of relays were not satisfactory. For very rapid switching, it would be necessary to use such devices, as the time to operate a relay was determined to be about 10 milliseconds. For times of the order of seconds (which we will probably continue to use), the positive feedback switching amplifier driving a control and switching relays is satisfactory.

b. The level of the forward and reverse current is now obtained from the taps of multiturn potentiometers between +15 or -15 volt supplies to ground. Turn-counting knobs will allow accurate setting and resetting of these voltages which are relay controlled inputs to the Driver amplifier.

c. The Blank Follower was changed to an inverter and the comparison amplifier then changed to a direct subtractor to obtain the control loop voltage. These changes get away from using the non-inverting inputs which give rise to non-linear gain problems.

d. The switching of the forward/reverse operation is now taken from the E-value and the switching amplifier is provided with multiturn potentiometers to give offset and bias settings so the switching occurs at determined values on either side of E 1/4.

e. A diode around the control relay lessens the switching transient when the relay goes inactive.

A circuit built for use as a constant potential coulometer can be used to subtract and integrate the cell currents. Further tests with the Photovolt Integraph show that it will count at the rate of 3800 cpm, which is limited by a selenoid digit counter counting the tens and higher places. This can be fed from the above amplifier setup and give digital data directly, but probably only for a single forward run.

3. Coulometry.

a. The coulometric titration of sodium phenobarbital. The reaction between sodium phenobarbital and mercury(II) can be used to quantitatively determine sodium phenobarbital. The product of the reaction is the mercury salt of phenobarbital, which is precipitated as a white precipitate.

The generation of Hg(II) can be accomplished anodically by making a mercury pool the anode of the coulometric cell. This was done in a modified Cotlove titration cell which has a total volume of approximately 5 ml. Approximately 1.5 ml of mercury is added to the bottom of the cell; electrical contact is made to the cell by sealing a platinum wire through the bottom of the cell. The amount of mercury added to the cell is not critical; it is simply added until the platinum wire is completely covered. The cathode is a platinum wire which is isolated from the cell by means of a KNO_3-agar salt bridge. The salt bridge is a glass tube which has been bent into a shape similar to the letter "N". A small amount of aqueous KNO_3 is added to the isolated portion of the salt bridge, and the platinum wire is allowed to sit in the solution of KNO_3. Stirring is accomplished by floating a magnetic stirring bar on top of the mercury.

The titration of sodium phenobarbital at levels of 2 to 10 microequivalents can be done in an aqueous solution of KNO_3. The procedure used was to add 3 ml of 0.67 M KNO_3 to the cell, giving a final molarity of 0.5 M. The standard solution of sodium phenobarbital is then added, along with an additional 2 drops of standard solution. The additional 2 drops of standard solution are also added to the blank solution so that an appreciable titration time can be obtained for the blank. The blank solution is identical to the standard solution, with the exception that water is added to replace the sodium phenobarbital. The final volume of the cell is about 4 ml. Some typical results are shown in Table 1.

Table 1

Titration of Standard Solutions of Sodium Phenobarbital

μeq. Added	Time (sec.)	μeq. Found	N*	Mg. Added	Mg. Found
10.00	111.2	9.19	1.09	2.54	2.34
	111.7	9.24	1.08		2.35
8.00	94.2	7.49	1.07	2.03	1.90
	94.2	7.49	1.07		1.90
6.00	77.9	5.86	1.02	1.52	1.49
	81.3	6.30	0.95		1.60
4.00	60.3	4.10	0.98	1.02	1.04
	60.2	4.09	0.98		1.04
2.00	41.1	2.18	0.92	0.508	0.554
	38.1	1.88	1.06		0.477

* N = number of μeq added/μeq generated. Generation current = 0.1 μeq/sec. Current sensitivity = 0.003 μA/cm.

It is seen from the value of N in Table 1 that the Hg(II) and sodium phenobarbital react in a one-to-one ratio. The reproducibility of the titration time is less than 3.5 sec and the accuracy ranges roughly from 1.5 to 8.0 percent. Linearity is observed over the entire concentration range.

In order to titrate samples less than 150 μg, a partially nonaqueous system was investigated. Two ml of 0.67 M KNO_3 was added to 1 ml of acetone. The final molarity after addition of the standard solution was 0.3 M in KNO_3 and the percent acetone by volume was 24%. Again, a spike was added to increase the blank titration. All other conditions were the same as before. In Table 3 are given the average values obtained, with the number of runs used to compute the average shown in parenthesis. In all cases the reproducibility is better than 3 sec, which corresponds to approximately one-half of a scale division on the chart paper. Qualitatively, the curves are the same as the aqueous curves at these same concentration levels.

At levels down to approximately 100 μg, the accuracy is better than 2.0 percent. Again, the ratio of Hg(II) to phenobarbital is one. Linearity of response is achieved over almost the entire concentration range.

A 48% acetone medium was next investigated at approximately these same concentration levels. Qualitatively, the curves obtained were the same as before, with the reproducibility better than 3 sec. The final KNO_3 concentration was 0.3 M.

The accuracy in 48% acetone is between 2-6%, even at levels of 70 µg. This appears to be the best medium for concentrations of this magnitude. One experimental disadvantage is present when working with acetone concentrations in this range. When acetone is present at about a 48% level, it has a tendency to dehydrate the salt bridge, leaving a vacant area where an air bubble possibly can form. This increases the resistance of the cell, and the coulometer cannot be balanced. If the salt bridge is inspected carefully before the titration is started, however, the air bubble can be removed and the titration can be completed without any difficulty.

b. The determination of salicylates in blood and urine. The currently accepted method of determining salicylates in body fluids is a spectrophotometric one. The highly colored complex formed by Fe(III) and salicylate, and the measurement of its absorbance at 540 mµ, provides a very sensitive, selective method. For micro and ultramicro determinations, a satisfactory degree of precision can be achieved without an extraction step.

A literature survey of the past twenty years discloses several applications of coulometric titrations to salicylates, though none are in body fluids. These methods were either acid-base titrations or bromine generation followed by back-titration of the excess of bromine with copper(I). It is possible, we believe, to extend this latter method to blood and urine in the level of concentration required. We are hopeful that a method can be perfected which will allow use of the extremely strong complex with Fe(III) to give the selectivity and uniqueness that the method should have. If this should prove impossible, a combination of thin-layer chromatography and the Br_2-Cu(I) titration will be investigated.

Some recent work has indicated that the micro IR reflectance spectrophotometric method of Beyermann (26) may be applicable to the determination of salicylates. It has been possible to obtain quite respectable spectra using a mirror of 2-mm diameter from 20 µg of sodium salicylate. Reproducibility is a problem due to the crystallinity of the material. An attempt is being made to standardize and reproduce a method of "suspending" the small crystals in a thin film of Nujol on the mirror. If this can be accomplished, we hope to compare the results of the coulometric technique with those obtained by the micro IR reflectance method.

c. The titration of uric acid. The product of the reaction between uric acid and iodine in alkaline medium has been reported to be alloxan. Alloxan, however, is very unstable in alkaline medium and undergoes a benzylic acid type rearrangement to alloxanic acid. Paper chromatography and thin-layer chromatography have indicated the presence of alloxan and alloxanic acid; however, an unidentified spot which seems to be a major product is present. This unidentified spot has not been matched with regard to its

mobility with any of the reference test materials, and is presently being investigated by isolating it from a preparative thin-layer plate.

Fast-sweep polarography has indicated the presence of a material which has the same half-wave potential as alloxan (1.80 V vs. SCE). Alloxan has a poorly defined UV maximum which could easily be obscured by traces of uric acid; however, it is easily reduced with cysteine to dialuric acid which has a peak at 270 mμ and an extinction coefficient of the magnitude of uric acid. In spite of this, we have not been able to demonstrate the existence of alloxan by use of this technique.

The enzymatic coulometric method previously reported was found to give poor titration breaks when fresh plasma samples were titrated. This was found to be due to unstable interfering substances present in fresh plasma, which are normally destroyed in storage. Ascorbic acid is the probable offender, since it undergoes a slow secondary reaction in a pH 10 buffer and is normally rapidly destroyed in plasma on storage. This slow secondary reaction, which tended to obscure the end point, could be reduced by chilling the titrate in an ice-water bath. A tentative enzymatic coulometric method is proposed.

The method used was as follows. Plasma or serum was incubated directly with the enzyme preparation. The samples were diluted in the following manner: (1) total titratable substances - 250 μl of plasma and 1 ml of pH 9.0 glycine buffer; (2) sample blank - 250 μl of plasma and 1 ml of enzyme preparation; (3) uricase blank - 250 μl of water and 1 ml of enzyme preparation; and (4) reagent blank - 250 μl of water and 1 ml of glycine buffer. These samples were incubated at 37°C for 45 minutes. At the end of this period, 3 ml of tungstic acid was added to remove the proteins by precipitation. The samples were centrifuged and 3 ml of the clear supernatant was taken for titration. This was placed in the titration cell, which was chilled by circulating ice water around the cell. To each of these solutions 1 ml of 5 M KI and 1 ml of pH 10.1 carbonate buffer were added, and the solution was titrated to a given current reading. The time was recorded in seconds. Titer is in units of mg% of uric acid.

$$\text{Titer} = ((a - b) + (c - d)) \times 0.04763^{*}$$

$$^{*}(0.001\ \mu\text{eq/sec} \times \frac{100}{0.1765}\ 1/100\ \text{ml} \times 0.08405\ \text{mg}/\mu\text{eq})$$

Since uric acid is only titrated at pH's greater than 8, it was felt that a non-enzymatic procedure might be developed if the interfering substances titrated at a lower pH but not at a pH of 10.

The method used is described below. The sample was diluted in the following manner: (1) sample - 250 μl of plasma and 4 ml tungstic acid and (2) blank - 250 μl H_2O and 4 ml of tungstic acid. The proteins were removed by centrifugation and 3 ml of the filtrate was used for the titration. This was placed in a titration cell which was chilled by circulating ice water around the cell. To each of the solutions 2 ml of composite

buffer was added and the solution was titrated to a definite current reading; at this point 1 ml of carbonate buffer was added and the solution was titrated again to the same current reading. The time for the second reading was recorded in seconds.

$$\text{Titer (mg\% uric acid)} = (a - b) \times 0.04763^{*}$$

* (same as enzymatic coulometric method)

Recovery experiments indicate that the UV-enzymatic procedure reported gives low results at high uric acid levels (93%). This is due to the method of precipitation which involves equal volumes of acetic acid and sample. Lower pH values may cause considerable loss depending on the dilution of the sample.

Results have indicated low blank values for non-uric acid titratable substances in the control sera which are frequently found in normal plasma or sera. In titrating samples with the pH differential technique, it is sometimes necessary to extrapolate lines to the "kick-off point," rather than titrating to a given current reading, due to differences in the slopes of the breaks of the blanks and unknowns. This can lead to errors of about 5% if not compensated for.

Some of the substances which have been reported to interfere with the colorimetric method of analysis include: Ascorbic acid, cysteine, ergothioneine, glutathione, Hemin, and sodium sulfide. Substances which had no effect at pH 10 include: Allantoin, alloxan, creatinine, cystine, methionine, phenol, tryptophane, and tyrosine.

Efforts were made to study the effects of the removal of the interferences in order to deal with a solution relatively free of interferences. H. T. Miles (30) reported the use of an ion-exchange resin which was selective for sulfhydryl groups. This consisted of mercuric acetate incorporated into a phenol-formaldehyde resin. This preparation was tried and it was found that, at levels where 90% of the uric acid was recovered, only 50% of the cysteine present was removed. It was noted that both ascorbic acid and sulfhydryl groups tend to react with ferric ion. Ferric ion was incorporated into a cation-exchange resin. It was found that this means was effective in removal of cysteine, ascorbic acid, and glutathione without loss of uric acid (around 90% recovery). Recoveries were better using tungstic acid filtrates of plasma samples.

It was found that certain reagents tended to give a substantial blank and were subsequently replaced. Sodium iodide probably has some sulfide due to the method of commercial preparation. This was replaced by potassium iodide. Higher molarities of glycine buffer tended to obscure the titration break, so it was replaced by a carbonate buffer.

In the catalytic reaction of uric acid with uricase, hydrogen peroxide is formed as one of the products. Hydrogen peroxide in alkaline medium

tends to react with uric acid also. Although this may be desirable since it accelerates its removal from the solution, it has an undesirable effect in that it also reacts with many of the interfering substances mentioned and would tend to give high results in the enzymatic coulometric method. Peroxides can be removed by the addition of catalase to the enzyme preparation. The concentration of catalase mentioned in the methods section prevented the reaction of hydrogen peroxide with many of the active interferences mentioned. Catalase was the least effective with cysteine (90% loss), but the concentration of cysteine normally present is small enough that it introduces little error into the method. Zinc has been reported to inhibit the enzyme reaction. It is normally present in a concentration of about 1 mg% in serum. Synthetic mixtures were prepared in a concentration ten times the normal value without any effect on the enzyme reaction. The enzymatic coulometric procedure was found to be useful in kinetic measurements of the uric acid reaction. To obtain accurate kinetic data on uric acid degradation, it is necessary to apply corrections for the UV absorption of an unstable intermediate. No corrections are necessary for this in the coulometric method. The overall time for the complete consumption of uric acid is "apparently" shorter since it is not necessary to wait for the complete decomposition of this unstable intermediate which is UV active.

Urine samples were run using the same type of titration procedures described in the methods section. The precipitation of proteins was eliminated in the pH differential method and the samples were diluted directly and titrated. The coulometric enzymatic procedure yielded low results; there were indications of losses in the incubation procedure. The results of the coulometric pH differential method and the colorimetric procedure were comparable; the latter was 3 to 5% higher in value.

4. Polarography.

A. Polarography of amino acids in dimethylsulfoxide (DMSO). Initial work has been done on the polarographic behavior of several amino acids in DMSO solvent. Tetraethylammonium perchlorate was used as the supporting electrolyte.

A vacuum distillation apparatus was constructed for distilling DMSO to produce DMSO which gave no polarographic waves in the usable potential range of 0.3 to -2.6 V (vs. aqueous SCE), and thus was suitable for polarographic work.

Eastman tetraethylammonium perchlorate (TEAP) was recrystallized twice from water and dried in a vacuum oven at 60°C for about 12 hours.

Several different designs of polarographic cell were tried; the cell which gave the most reproducible and suitable results was a simple H-cell, with a medium porosity glass frit and an agar plug separating the test compartment from the aqueous saturated calomel reference electrode. Judging by the size of the reduction wave due to water at about -2.0 V, water seepage into the DMSO solution in the test compartment is not a serious

problem in terms of obscuring waves. Cell resistance was found to be relatively constant over several hours at about 1900Ω. The temperature of the cell was regulated at 25.0 ± 0.5°.

All solutions used for polarographic analysis were deaerated with nitrogen, which was scrubbed in one tower containing vanadium(II) and in a second tower containing DMSO. Nitrogen was passed over the test solution while the polarogram was being taken.

The polarography of β-mercaptoethylamine·HCl in 0.1 M TEAP in DMSO was studied. Waves were observed at about -0.17 V (anodic), and -1.8, -2.0, and -2.3 V (all cathodic). These results agreed quite well with previous studies of this compound under these conditions.

The following amino acids were studied in 0.1 M TEAP in DMSO: dl-methionine, l-thyroxine, l-tryptophan, and dl-alanine. The alanine required several days to dissolve and, during this time, the solution picked up enough water to lower the sensitivity with which the polarographic measurements could be made. l-Histidine did not dissolve in DMSO, even after standing for one week. Analysis of the data for the above compounds has not been completed, thus the results given in Table 10 are only approximate values for the half-wave potentials.

Table 10

Approximate Half-Wave Potential Values for Several Amino Acids

Amino Acid	Approximate $E_{1/2}$ (volts)
dl-methionine	-2.40
dl-alanine	-2.40
l-tryptophan	-2.40
l-thyroxine	-1.20
	-1.57
	-2.02
	-0.05 (anodic)

b. The polarography of vanadium in dimethylformamide (DMF). This investigation originally started as a polarographic study of the possible complexing between vanadium and several of the precursors of cholesterol. Because of the insolubility of most of these precursors in water, it was necessary to go to an organic solvent, DMF. At that time, vanadium had not

been studied in DMF. Previous work on the polarography in DMF has employed LiCl as the supporting electrolyte and a mercury pool reference electrode. In the present instance, this course proved to be unsatisfactory. A wave was obtained at -0.26 V, but the potential of this wave varied by tenths of a volt and the pool had to be changed often.

A reference electrode that had also been used for the study of inorganic ions in DMF was the aqueous saturated calomel electrode. The Junction potential between the two solvents was compensated by using a three-electrode system. Marple suggested that a $CD_{(HG)}$, $CdCl_2$ electrode in DMF might serve as a suitable reference in this solvent. Several of these electrodes were made up in H-cells and their leads were shorted. After one day, the electrodes were found to have achieved the same potential to within several tenths of a millivolt.

To avoid contamination of the cadmium reference electrode, a three-compartment H-type cell was designed. The glass frits separating the compartments were so chosen that when a solution of potassium permanganate was placed in the center compartment, no pink color would appear in the other two compartments within 25 minutes. The center compartment was kept empty except during a run and was immediately emptied after each run.

After the three-compartment cell was designed, a great deal of time and effort was expended on getting the DME to work properly. The best results were finally obtained with a drawn electrode where the tip had smaller bore than the rest of the capillary. In the absence of this drawn capillary, some DMF tended to rise into the bore where it reacted with mercury, causing erratic drop times and totally incomprehensible waves.

The supporting electrolytes used have been LiCl, tetraethylammonium chloride, tetraethylammonium iodide, and tetraethylammonium perchlorate. In the first two cases, the chloride ion complexed with the vanadyl ion, greatly changing the $E_{1/2}$ and the current. If a vanadium-LiCl solution is made up in DMF and polarograms are run at different time intervals, the wave disappears with time. If the concentration of vanadium is decreased from 1 molar to 0.1 molar, the $E_{1/2}$ shifts more positive by about 0.3 V. In addition, there was a real problem in maintaining the same potential with the LiCl supporting electrolyte.

Tetraethylammonium iodide reacts with the solution forming free iodine. The solution gave a positive starch test.

To date, tetraethylammonium perchlorate has given good results. The only drawback seems to be that the cell has a resistance of 2500 ohms. A three-electrode system has not been used because the concentration of vanadium that gives a wave without a maximum has a diffusion current of only about 1.5 μA; this would alter the potential by 3.7 mV. At present, the resolution of the half-wave potential has been less than this value.

To date, the greatest problem has been with the determination of vanadium in DMF. Presently a 0.0862 M aqueous solution of the vanadium is added to the DMF solution of TEAP. Three-tenths ml of the vanadium is diluted with 10 ml of the DMF making the analyzed solution 2.5×10^{-3} M in vanadium. A critical question is whether the presence of water effects the half-wave potential of the vanadium. Some studies have been made on this subject, but no conclusive evidence has been obtained. Michlmayr and Gutmann state that water has no effect on the half-wave potential up to a water concentration of 4%.

The wave obtained in DMF with the TEAP supporting electrolyte is quite drawn out, extending over 0.75 volt and with a half-wave potential of -0.370 V. Due to the small slope of the polarogram, the half-wave potential could not be determined to better than 8 mV. No other waves were noted in the polarogram.

In order to study the electron change, a constant potential coulometer was built using a design of Underkofler and Shain. This coulometer employs a three-electrode design which necessitated the development of a workable $Cd_{(Hg)}$, $CdCl_2$ electrode. The final design of this electrode is shown in Figure 6. Four of these electrodes were made and their leads were then connected and the electrodes were allowed to stand overnight in a TEAP solution. The leads were then disconnected and after three hours the potential differences were measured. The two electrodes with the smallest potential difference were then compared to the reference electrode of the polarograph and the one that agreed best with this latter electrode was used in the coulometer. After several days of use, the potential of this electrode had only changed by 4.14 mV.

The concentration of the vanadium in the aqueous solution was determined by a permanganate titration at 80°C. Using this concentration data and the number of coulombs passed by the coulometer, an n-value of 1.47 was calculated. This value is probably a combination of the coulometer operation and the difficulty in the polarographic determination of the unreacted vanadium. Needless to say, this coulometric work will be repeated.

Another difficulty encountered is that to date our work does not agree with that of Michlmayr and Gutmann. These two workers studied the polarography of vanadium and chromium in DMF and DMSO. They employed VCl_3 and $VO(ClO_4)_2$ as sources of vanadium. For the reduction of vanadium, these workers obtained three waves at -0.64, -1.76, and -2.52 V vs. SCE. The only difference between the operating procedures was that they used an SCE as reference. We are currently trying to repeat the work of Michlmayr and Gutmann for we have encountered some discrepancies in their work. We also intend to prove the usefulness of the cadmium reference electrode mentioned above.

Summary and Conclusions.

Initial studies have begun on the use of atomic absorption spectroscopy for the determination of vanadium in biological materials. Chronopotentiometric instrumentation has been designed employing a two-cell system for the compensation of the normal charging current in electrode processes. The capability of cyclic work has been built into the instrument and instrumental parameters have been checked out with test solutions of cadmium ion. Coulometric titrations have been applied to the determination of sodium phenobarbital. As little as 70 μg can be titrated with mercuric ion in an electrolyte which is 48% acetone and 0.3 M in potassium nitrate. The accuracy of the determination is between 2 and 5%. Preliminary work on the determination of salicylates has begun using an indirect bromine titration. Uric acid in 250 μl of plasma or serum is determined either by an enzymatic or a pH differential determination. The titrant, iodine, reacts with uric acid and other iodine-titratable substances in the sample. The polarography of several amino acids has been investigated in the solvent DMSO. Also, the polarographic behavior of vanadium in DMF has progressed. One of the major problems, that of the reference electrode, has been overcome by the design of a cadmium amalgam-cadmium amalgam-cadmium chloride electrode made up in the solvent. (U)

Publications.

Simon, R. K., Christian, G. D., and Purdy, W. C. Coulometric determination of arsenic in urine, Am. J. Clin. Path., 49, 207 (1968).

Simon, R. K., Christian, G. D., and Purdy, W. C. Comparison of methods for the elimination of organic matter in human urine. Application to toxicological samples, Am. J. Clin. Path., 49, 733 (1968).

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RESEARCH AND TECHNOLOGY RESUME

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(U) APPROACH- TO RELATE X-RAY STRUCTURE ANALYSIS TO SPECIFIC CONFIGURATIONS OF THE CHEMICAL MOLECULE WHICH ENHANCE PROTECTIVE CAPACITY OF THE CHEMICAL.

25. (U) PROGRESS - JUN 67 THRU 30 JUN 68 THE RESULTS OF INVESTIGATION OF THE PRECISION AND ACCURACY OF THE WALTER REED DIFFRACTOMETER BY USE OF A 50-MICRON NACL CRYSTAL HAVE BEEN ANALYZED. BEST RESULTS WERE OBTAINED WITH THE LARGEST CRYSTAL THAT WILL BE BATHED IN THE HOMOGENEOUS X-RAY BEAM. DIFFRACTION MAXIMA MUST BE INVESTIGATED AT LEAST TWICE. NO SERIOUS SYSTEMATIC ERRORS WERE NOTED, THE FINAL ERROR WAS 4 PERCENT IN THE INTENSITIES MEASURED FOR NACL. ALL DATA ON 2-(2,4. DIMETHYL PHENYL-3-METHYL 6-CHLORO-7,8-BENZOQUINOLINE-4-CARBOCYCLIC ACID METHYL ESTER (ARMONE) HAVE BEEN TAKEN. THE SCATTERED INTENSITY DATA FOR ARMONE HAS BEEN GATHERED AND PROCESSED THRU STAGES OF CALCULATION USING THE X-RAY 67 SYSTEM OF CRYSTALLOGRAPHIC PROGRAMS. PROGRAMS SIGMA 2 AND PHASE WERE USED TO FIND AND SOLVE FOR SIGNS RELATIONSHIPS. RESULTANT E MAP YIELDED A TRIAL STRUCTURE WHICH PROVED CORRECT AND IS BEING REFINED. MAIN FEATURES OF CHEMICAL STRUCTURE OF THIS COMPOUND HAVE BEEN CONFIRMED. EARLY THREE-DIMENSIONAL MAPPING IS ANTICIPATED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House laboratory independent research

Work Unit 175, X-Ray diffraction studies of biological interest

Investigators.

Principal: James Stewart, Ph.D.
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COL E. C. Knoblock, MSC

Associate: Professor Roger Chastian, Jr., Ph.D.
Professor Herman Ammon, Ph.D.
Mrs. Linda Plastas, B.A.

Description.

This program was initiated to apply the sophisticated X-ray crystallographic techniques to the detailed study of structure of biochemical compounds and to organic structures in order to get more specific chemical data to relate to in vivo and in vitro reactions of these compounds. Data provided will not only define the internal geometry of the agents studied, but comparisons with closely related structures and optical isomers of the same chemical agents will be utilized to gain further insight into the specificity of these molecular species in the biochemical system and will assist in explaining some of the observed phenomena of structure-activity relationships of drug activities. The initial studies will include a series of antimalarial agents (the quinolines) which have side effects of varying degrees of photosensitization.

Progress.

1. Preliminary use of the equipment. During the first year the WRAIR diffractometer was used extensively. Every effort was made to assure that the experimental techniques necessary to realizing its potential for producing precise molecular geometry of the antimalarials would be realized. To this end data were gathered for eight compounds, three of which had been previously gathered either photographically or on another diffractometer. These were only qualified successes but served to give experience which could then be applied in the gathering of the data for ARMONE. There also were a number of complications derived from the complexity of the automation of the diffractometer itself. Professor H. Ammon of the University of California at Santa Cruz and Professor R. Chastain of the West Virginia University both made significant contributions in the preliminary alignment, programming for and testing of the instrument.

2. Thorough check-out of the equipment. By the time Dr. E. Boonstra arrived in September it was clear that a careful run of a well-known substance must be made. Sodium chloride was chosen and a small crystal selected

and mounted. The description of the results of that work is given in the next section. The instrument consists of six major parts:

Thermostated room
Line regulating power source
Highly regulated x-ray source
X-ray detecting and counting instruments
Eulerian crystal mount with four independent angular adjustments
Datex automation to control from punched cards, three angles, the shutter, and the x-ray counting equipment

The first two items are provided by the university. They have been excellent in performance and have led to very great stability in the x-ray source and counting circuits. The General Electric x-ray source and count- equipment have also been very good.

The first major problem we encountered was the exact alignment of the Eulerian cradle with respect to the x-ray source. This proved to be a very time-consuming process the first time it was undertaken (two-man months). However, once accomplished, the cradle itself proved to be excellent save in the inordinate backlash in one angle (ϕ). We have solved this problem by always sorting input data cards to drive ϕ forward to its setting position.

It is with the Datex automation that we have had the greatest problems. The unit is marginal and, as I reported to Colonel Knoblock during the course of the year, the service has not been perfect. After his strongly worded request for service was sent to General Electric, and then to Datex, we have been running much better, but we seriously hope that a new maintenance contract will be negotiated for the instrument.

At this writing we are confident that the machine produces good diffraction data. It is essentially automated, but the Datex equipment must be watched continually and serviced more often than we had hoped. The most serious problems were those associated with our own understanding of the techniques of diffractometry. The next section describes the work done to overcome this difficulty. Dr. Boonstra has added the following information:

The preliminary experience obtained by using the diffractometer as described in 1, and the difficulties that were encountered, showed that it was necessary to check both the equipment and the experimental techniques carefully.

The diffractometer was carefully realigned in the way suggested by the manufacturers and no serious misalignment was noted. It is possible though that a number of small adjustments could have an appreciable effect in some measurements.

The counting circuits were checked, especially the linearity of the counter, which was satisfactory. The stability and reproducibility of these circuits were judged good.

The encoder motors on the three angular drives, although satisfactory on the whole, did occasionally give trouble. Sometimes, e.g., the ϕ drive would stick or hunt in a definite angular range, which could usually be obviated by cleaning and lightly regreasing the slide. The backlash on the ϕ drive is a direct result of the mechanical drive which had to be used, and consequently all ϕ settings are always made with ϕ increasing. The 2θ drive occasionally stuck due probably to a high spot on the drive surface and excessive drag produced by the cables. The suspension of the cables was corrected to decrease this effect. Any irregularity in the 2θ drive is extremely undesirable since it affects intensity measurement using the 2θ scan method. It was sometimes noted that the scan times over a constant angular range did sometimes vary by 1 or 2 secs on 50 secs, and it is obviously important to check this periodically to ensure that any experimental variation is within the bounds of the desired accuracy.

On the whole, the equipment described thus far operated satisfactorily. Unfortunately, in the course of the year, a lot of trouble was encountered in the use of the card reader, card punch, and their interaction with the Datex unit. This aspect has been discussed previously and has been attended to so that no further report is made here.

3. Use of NaCl as a test standard. The technique of intensity measurement was tested on single crystals of NaCl. The crystals used were small (about 50 micron cubes) and the results showed that although such small crystals could be used for alignment, larger crystals (bearing in mind that absorption only as a secondary or tertiary effect) should be used in order to increase the diffracted intensity and hence the statistical accuracy of the measurement. Of course, the size of the crystal should not exceed the homogeneous region of the incident x-ray beam.

The measurements on NaCl were confined to the $2\theta:\omega$ scan method. Since then it has become clear that the peak height method, coupled to a proper calibration curve, should under most circumstances be a more efficient way of intensity measurement.

Intensities were measured for a complete hemisphere in reciprocal space and, on account of the high symmetry, these contained many equivalent intensities. In order to compare agreement between equivalent reflections as a function of the different variables, a special program DFTEST was written. The average agreement between equivalent intensities varied from 2 to 4%, being better for the strong (even) reflections, except for the very strong (200) reflection which apparently is affected by extinction. The most obvious correlation is between accuracy and intensity, or in this case accuracy and angle diffraction. For this reason it is believed that crystals as large as possible should be used in data collection. No systematic errors in the equipment, say a dependence of intensity or accuracy on,

e.g., ø, could be observed.

After averaging the measured intensities of equivalent reflections refinement of the scale factor, the individual isotropic temperature factors for Na^+ and Cl^- yielded an R factor of 0.02. In agreement with the literature, the actual values of the temperature factors depend on the individual crystal.

The process of averaging which is needed to produce this low R value indicates that in an ordinary structure determination it would be advisable to measure not only the asymmetric set of reflections, but at least one other equivalent set. When two such measured intensity values agree to within one or two standard deviations, a good average value is available. If the one measurement is lower, the other (i.e., the higher) measurement should usually be taken, but if the disagreement is large, the reflections concerned should be remeasured and the discrepancy investigated until resolved satisfactorily.

4. Development of crystallographic programs. Two types of programs are necessary for the solution of structures utilizing the Army diffractometer. The second type is that used in the calculations which transform the information contained in the x-ray intensities to structural parameters (e.g., the accurate atomic coordinates of the molecules which constitute the crystal). All the programs used have been developed over a long period of time under support from NASA Grant NsG-398 to the Computer Center of the University of Maryland. The results produced on ARMØNE are the results of the first use of the latest versions of these programs which is called X-Ray-67. All the work done was carried out on the UNIVAC 1108 under EXEC VIII. It is felt that the work done represents a major step in establishing and proving codes which may now be used routinely to carry out structure determinations. We now have in hand, among others, the following operational programs that were used in the solution of this structure.

DIFSET	used to generate the Army diffractometer setting.
PARAM	used to establish accurate unit cell lengths and angles.
DATCØ3	used to process the output from the diffractometer.
DATRON	used to prepare the intensity cell and symmetry data for all further calculations.
DATFIX	used to estimate scale and temperature parameters and determine values of normalized structure factors (E).
SIGMA2	used to determine all the structure invariants among the data preparatory to the solution for the phases.
PHASE	used to find the phases of the largest normalized structure factors.

FOURR — used to carry out the Fourier transformation from E to probable location of atoms in the unit cell (E MAP) and later to calculate electron density maps of the structure.

FC — used to calculate from the trial structure the structure factors and their phases.

ØRFLS — used to produce by least squares the accurate atomic coordinates of the structure.

BONDLA — used to determine the interatomic distances and their estimated standard deviation.

LSQPL — used to determine the least squares planes formed by atoms in the molecule and angles between these phases.

5. Preliminary Structural Data for ARMØNE, the methyl ester derivative. Crystals of this compound (2-(2-4-dimethylphenyl)-3-methyl-6-choloro-benzo (h) quinoline-4-carboxylic acid methyl ester) were prepared and supplied by CPTs Rubin and Rice. A photographic investigation showed that the structure is monoclinic, $P2_1/C$, the approximate cell dimensions were determined and a suitable crystal selected for use on the diffractometer. This is important, since some crystals were found to be twinned, and could severely complicate the interpretation of data collected on the diffractometer.

The unit cell dimensions as measured on the diffractometer and obtained from a least squares fit are:

$a = 11.993 \pm 0.003$

$b = 5.2379 \pm 0.0008$

$c = 30.583 \pm 0.009$ Å

and $\beta = 90^{\circ}39 \pm 2'$

where the errors indicated are e.s.d. There are four molecules per unit cell.

Over a two-week period, intensities were measured on the diffractometer for nearly 5000 reflections using the $2\theta:\omega$ scan technique with a scan speed of 2° per minute, a constant scan width of 1.7° 2θ, 40 second background counts on both sides of the peak, and employing MoKα radiation. Of the 4609 unique reflections, 2147 had measured intensities above the 1-sigma level and only 1025 above the 3-sigma level, where sigma is the e.s.d. of the measured intensity.

The data were processed to yield for each reflection the quazi-normalized structure factor E, since the aim was to use a direct method of phase determination to solve the structure, which is centro-symmetric. A total of 826

reflections is found to have E values above 1.30, and the sigma2 relationships for these reflections were obtained with the new program SIGMA2. More than 30,000 potentially useful relationships were found. Since the structure contains 28 atoms (non-hydrogen), there are 84 positional parameters to be determined. Hence, the program PHASE was used to determine a solution with 85 generators. A very promising solution was readily obtained which gave the signs of 529 phases. On this basis an E map was calculated and inspection promptly revealed the position of all the atoms in the molecule.

This trial structure was refined to an R value of 10.4%, using isotropic individual temperature factors and unit weights for all the reflections above the 3-sigma level. Further refinement, after the introduction of anistropic temperature factors and a suitable weighing scheme, should decrease the R factor and improve the atomic positions and their accuracy. This will be done as the next step.

The available data on the molecular structure (all obtained from the atomic positions corresponding to R - 10.4%, as described above) are summarized on the four figures attached. The bond lengths and angles are all reasonable with the possible exception of the bonds C15-C25 and C25-C26. The main feature of the structure is that the benzoquinoline system which consists of three rings is approximately planar, but the three rings seem to be inclined to each other at angles from 2 - 6°. At the present level of refinement no speculation on this would be justified.

Another important feature is that the dimethylphenyl ring is planar and rotated out of the plane of the benzoquinoline system at an angle of 66° (± 3°) about the C12-C41 bond. Similarly, the carboxylic acid methyl ester group (C18, O1, O_2 and C19) is planar and rotated out of the benzoquinoline plane by 75° (± 3°) about the C14-C18 bond.

At the present time, the packing of the molecules and the possible existence of hydrogen bonding, or other interesting features, have not yet been investigated.

Summary and Conclusions.

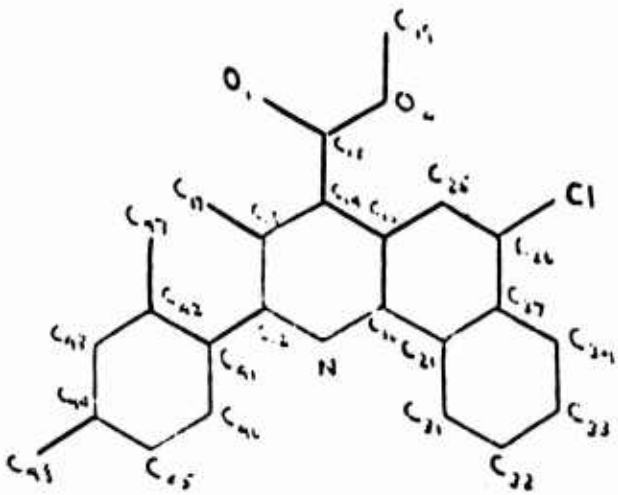
The x-ray diffractometer has been assembled and fully standardized for crystal studies. The equipment has operated satisfactorily with the exception of the card-reader - card-punch assembly. The difficulty in this regard has been overcome. Operational computer programs have been developed and used in the solution of crystal structures. The first of the antimalarial compounds (ARMØNE) has been completely analyzed. The three-dimensional structure of this compound is currently in the final analytical stage. The data provided indicate that the benzoquinoline structure of three rings is planar. The dimethylphenyl ring is planar and rotated out of the plane of the benzoquinoline system, as is the carboxylic acid methyl ester group. Bond angles have been defined for these rotations.

Publications.

None.

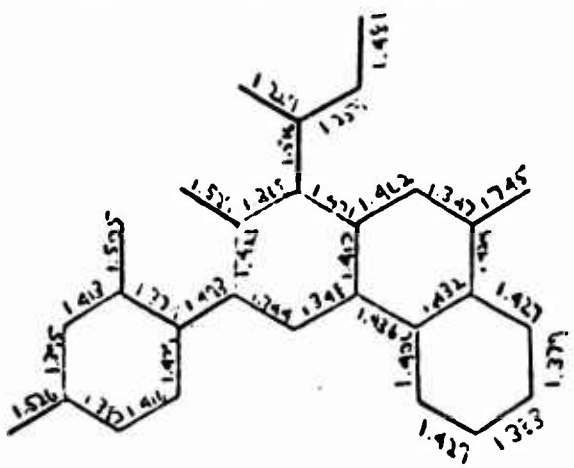
2-(2-4-DIMETHYLPHENYL)-3-METHYL-6-CHLORO-BENZO(h)QUINOLINE-4-CARBOXYLIC ACID METHYL ESTER (ARMONE or PMDQ)
ATOMIC NUMBERING SCHEME 5 JUNE 1968

FIGURE I



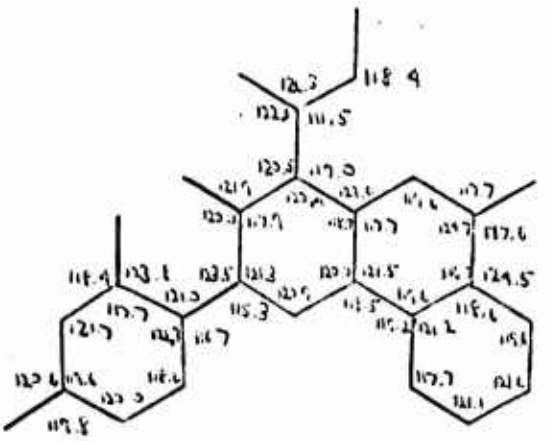
ARMØNE (PMDQ) BOND LENGTHS IN Å (e.s.d. ~ 0.01 Å)
(ISOTROPIC REFINEMENT UNIT WEIGHTS R = 10.4%) 5 JUNE 1968

FIGURE II



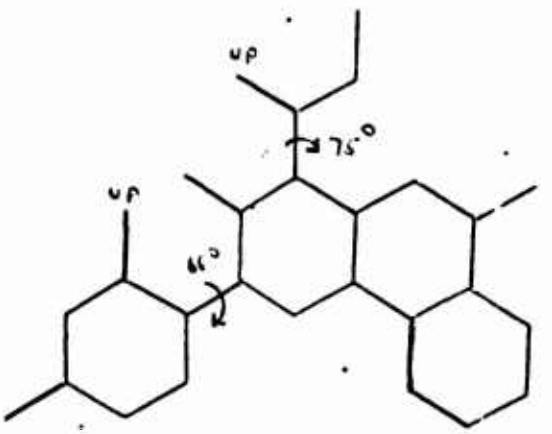
ARMØNE (PMDQ) BOND ANGLES (e.s.d. ∿ ·7°)
(ISOTROPIC REFINEMENT UNIT WEIGHTS R = 10.4%) 5 JUNE 1968

FIGURE III



ARMØNE (PMDQ) TWISTS ON BONDS REQUIRED TO TURN PLANAR GROUPS OF IDEALIZED MODEL TO GIVE APPROXIMATE STRUCTURE OF MOLECULE

FIGURE IV



RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CR6433	CSCRD-102

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

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 176

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) FIELD EPIDEMIOLOGY SURVEY TEAM

12. SCIENTIFIC OR TECH. AREA: 003500 CLINICAL MEDICINE 005900 ENVIRONMENTAL EIC

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
07 66	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. TYPE NA b. NUMBER NA c. DATE NA d. AMOUNT NA	PRIOR FY 68	2	110
		CURRENT FY 69	2	110

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

RESP. INDIV: MERONEY, COL W. H.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION
NAME: WRAIR TEAM
ADDRESS: VIETNAM

INVESTIGATORS PRINCIPAL: WEBB, LTC C.
ASSOCIATE: LEGTERS, LTC L.
TEL: 202-576-3384 TYPE: DA

21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NA

23. KEYWORDS: EPIDEMIOLOGY, TROPICAL DISEASES, VECTORS.

24.

(U) TECH OBJECTIVE - TO DETERMINE THE FEASIBILITY AND THE PRODUCTIVITY OF A SPECIAL FORCES MEDICAL EPIDEMIOLOGICAL TEAM WORKING IN A FORWARD COMBAT AREA.

(U) APPROACH- TO TRAIN, EQUIP, AND DEPLOY SUCH A TEAM OF 8 MEDICAL OFFICERS, 3 MSCS AND 16 EM TO VIETNAM.

(U) PROGRESS - JUL 67 THRU JUN 68 THE TEAM HAS BEEN FUNCTIONING. PRELIMINARY DATA INDICATE THAT THIS APPROACH IS USEFUL IN HIGHLY SPECIALIZED SITUATIONS. WHILE THE SPECIALIZED MILITARY QUALIFICATIONS ARE VERY USEFUL UNDER COMBAT AND NEAR COMBAT CONDITIONS, IN OTHER SITUATIONS THESE QUALIFICATIONS ARE NOT REQUIRED AND INDEED MAY HAMPER DEPLOYMENT OF THE TEAM TO CERTAIN COUNTRIES. FOR TECHNICAL REPORTS, SEE THE ANNUAL REPORT OF THE U.S. ARMY MEDICAL RESEARCH TEAM (WRAIR) VIETNAM.

26.

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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

RESEARCH AND TECHNOLOGY RESUME

1.	2. DOVE ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 086434	CSGRD-103

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

10. CURRENT NUMBER CODE
6113001I 3AC13001A91C 00 177

10a. PRIOR NUMBER CODE

11. TITLE (U) TROPICAL DISEASE BULLETIN INFORMATION RETRIEVAL SYSTEM

12. SCIENTIFIC OR TECH. AREA		13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002600 BIOLOGY	004200 COMPUTERS	08 67	NA	OTHER DA

16. PROCUR. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	08 67			
B. CONTRACT	b. NUMBER DADA17-68-C-8012	PY 68	1	22
	c. TYPE h.LH d. AMOUNT $23,220	CY 69	1	23

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

RESP. INDIV. MERONEY, COL H. F.
TEL 202-576-3551

20. PERFORMING ORGANIZATION
NAME COMPUTER APPLICATIONS, INC.
ADDRESS 8555 16TH STREET
SILVER SP MD 20910

INVESTIGATORS PRINCIPAL VOCCOLA, H. h.
ASSOCIATE JACOBUS, D. P.
TEL 202-576-2280 TYPE UP

21. TECHNOLOGY UTILIZATION
PHARMACEUTICAL INDUSTRY

22. COORDINATION
NA

23. KEYWORDS
MALARIA, INFORMATION HANDLING, ABSTRACTS, COMPUTER PROGRAMMING, INFORMATION RETRIEVAL.

24

(U) TECH OBJECTIVE - THE OBJECTIVE IS TO DEVELOP A COMPUTER SYSTEM TO CREATE, MAINTAIN AND INDEX THE ABSTRACTS FROM THE TROPICAL DISEASE BULLETIN. SINCE THE SIZE OF THE FIELD OF EACH ITEM IN THE TROPICAL DISEASE BULLETIN SOURCE FILE VARIES, THE RESULT OF THIS OBJECTIVE WILL BE A TROPICAL DISEASE BULLETIN MASTER FILE IN A FORMAT COMPATIBLE WITH OTHER EXISTING RETRIEVAL SYSTEMS. A SECOND OBJECTIVE WILL BE TO DEVELOP MANIPULATIVE CAPABILITIES SUCH AS THE UPDATING OF THE MASTER FILE AND SORTING ON CERTAIN FIELDS WITHIN THE FILE.

25 (U) APPROACH- THE PROBLEM ORIENTED TEMAC LANGUAGE WILL BE USED BECAUSE OF THE ABILITY TO HANDLE VARIATIONS IN SPELLING OF WORDS AND PUNCTUATION.

(U) PROGRESS - JAN 68 THRU JUN 68 DURING THE PAST YEAR PROGRAMS IN TEMAC AND MAP HAVE HAVE BEEN COMPLETED IN ORDER TO BUILD AND CORRECT THE FILE CREATED IN THE TROPICAL DISEASE BULLETIN PROJECT. THESE PROGRAMS HAVE INVOLVED DETECTION OF ERRORS AND INSERTION OF CORRECTIONS AS WELL AS COMPILING THE INPUT TAPES INTO AN APPROPRIATE MASTER FILE. IN ADDITION, PROGRAMS HAVE BEEN COMPLETED FOR CONVERTING TO BIOLOGICAL ABSTRACTS THE SUBJECT, TITLE AND AUTHOR INDICES SO THAT THE EXISTING PROJECT EXPERT SYSTEM CAN HANDLE THE TROPICAL DISEASE BULLETIN. IN ADDITION, SEARCH QUESTIONS INDICATING WORDS OR PARTS OF WORDS HAVE BEEN SUCCESSFULLY RUN ON EITHER THE FULL TEXT OR THE TITLES OF TROPICAL DISEASE BULLETIN. THE FUTURE WORK VISUALIZES COMPRESSING THE EXISTING FILE TO REDUCE THE SEARCH TIME, THE PROVISION OF OR QUESTIONS AND THE DEVELOPMENT OF A ROUTINE INQUIRY SYSTEM CAPABLE OF BEING OPERATED BY A NON-PROGRAMMER. THIS SYSTEM WILL PROVIDE TO THE INQUIRER APPROPRIATE COMMENTS ON SYSTEM USES. IF THE PROJECT DEVELOPS AS EXPECTED, THE EXISTING SEARCH TECHNIQUES AND MASTER FILE WILL BE USED TO DEVELOP MEASUREMENTS TO ESTABLISH THE BASIS FOR PROGRAMS ON THE NEW WRAIR MACHINE PERMITTING THE DISCOVERY OF SYNONYMS WITHOUT THE USE OF AN EXISTING THESAURUS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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.

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

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 177, Tropical Diseases Bulletin
Information retrieval system.

Investigators:

Principal: Harry W. Voccola

Description

The purpose of this effort is two-fold. The first objective is to develop the software capable of handling the input created by the encoding of the Tropical Disease Bulletin and to manipulate it so as to correct errors, build the file, reformat index tapes suitable for handling by the regular Biological Abstracts system. The second objective is to develop a search technique capable of handling full text, fractions of words within text, and ultimately, manipulation procedures involving the discovery of synonyms without the use of a thesaurus.

Progress

During the past year programs in TEMAC and MAP have been completed in order to build and correct the file created in the Tropical Disease Bulletin project. These programs have involved detection or errors and insertion of corrections as well as compiling the input tapes into an appropriate master file. In addition, programs have been completed for converting to Biological Abstracts the subject, title, and author indices so that the existing Project EXPERT system can handle the Tropical Disease Bulletin. In addition, search questions indicating words or parts of words have been successfully run on c.ther the full text or the titles of Tropical Disease Bulletin. The future work visualizes compressing the existing file to reduce the search time, the provision of OR questions, and the development of a routine inquiry system capable of being operated by a non-programmer.

Summary and Conclusions

This work has made good progress in that there is now a searchable file using relatively simple search strategies. The introduction of more sophisticated techniques is to be expected during the forthcoming year. These techniques are expected to lead to a general searching capability capable of handling relatively unpurified text. Such a capability will be of broad interest to the Army Medical Service.

Publications

None.

RESEARCH AND TECHNOLOGY RESUME	1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
			DA 686430	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 69	D. CHANGE 01 10 67	U U	NA	DC	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 178

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) PROGRAMMED THAI FOR MILITARY MEDICAL PERSONNEL

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
009000 LINGUISTICS	10 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	b. NUMBER		10 67			
B. CONTRACT	c. TYPE	DADA17-68-C-8049 P.CPFF	d. AMOUNT $65,355	PRIOR FY 68	1	65
				CURRENT FY 69	1	65

19. GOVT. LAB/INSTALLATION/ACTIVITY	20. PERFORMING ORGANIZATION
NAME: WALTER REED ARMY INST OF RES	NAME: INST FOR BEHVAL RSCH, INC.
ADDRESS: WASHINGTON D C 20012	ADDRESS: SILVER SP MD 20910
RESP. INDIV.: PERONEY, COL W. F.	INVESTIGATORS PRINCIPAL: HUGHES, H. B. PH.D
TEL: 202-576-3551	ASSOCIATE: BRADY, COL J. V.
	TEL: 301-587-2909 TYPE UP

21. TECHNOLOGY UTILIZATION	22. COORDINATION
BEHAVIORAL SCIENCES	NA

23. KEYWORDS: PROGRAMMED THAI, MILITARY MEDICAL PERSONNEL, VERBAL BEHAVIOR.

24.

(U) TECH OBJECTIVE - TO ASSESS THE TRAINING VALUE OF AN EXPERIMENTAL COURSE IN PROGRAMMED THAI FOR MILITARY MEDICAL PERSONNEL, TO MAKE SUCH REVISION IN TECHNICAL FEATURES AND CONTENT THAT MIGHT BE REQUIRED, A SYSTEMATIC BEHAVIORAL ANALYSIS OF VERBAL INTERACTION BETWEEN MILITARY MEDICAL PERSONNEL AND THEIR THAI COUNTERPARTS.

(U) APPROACH- APPLICATION OF A MATCHING TO SAMPLE TECHNIQUE WHEREBY SPEAKING AND WRITING SKILLS ARE TAUGHT BY SHAPING, LISTENING, AND VIEWING DISCRIMINATIONS, THROUGH A COMPLETELY AUTOMATED METHOD INVOLVING CODED FILM STRIPS AND SYNCHRONIZED AUDIO-TAPES, PRESENTED WITH AN AUDIO-VISUAL DEVICE THAT ALSO PERMITS ELECTRONIC RECORDING OF RESPONSES.

(U) PROGRESS - OCT 67 THRU JUN 69 AN EXPERIMENTAL COURSE IN PROGRAMMED THAI COMPRISED OF 40 SECTIONS HAS BEEN DEVELOPED AND IS BEING FIELD-TESTED AT THE SEATO LABORATORY-IN BANGKOK, THAILAND AND LABORATORY-TESTED AT THE INSTITUTE FOR BEHAVIORAL RESEARCH IN SILVER SPRING, MARYLAND. IN ADDITION, A STUDENT MANUAL, INCLUDING TEST EXERCISES, GRAMMATICAL SUMMARIES, AND VOCABULARY FOR EACH SECTION, HAS BEEN COMPLETED AND A SEPARATE DICTIONARY HAS BEEN MADE AVAILABLE TO COMPLEMENT THE AUTOMATIC INSTRUCTIONAL PROGRAM. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

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

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 178, Programmed Thai for military medical personnel

Investigator.
Heidi B. Hughes, PhD

Description.

The investigations conducted in conjunction with this work unit are concerned with the research development and experimental testing of an automated instructional program in Thai language skills for military medical personnel. The efficacy of some programming features commonly advocated as advantageous in foreign language learning such as self-pacing, immediate feedback, and disposition of errors is also under evaluation in relationship to the teaching material developed for this research project.

Progress.

Work on this project to date has progressed through the preliminary program development stages and is currently concentrating on testing the training value of the autodidactive Thai course. Completed research has made available 1) an audio-visual linear program consisting of 40 sections involving a vocabulary exceeding 1,300 words, 2) a student manual comprised of complementary instructional and evaluative material, and 3) a dictionary providing English/Thai and Thai/English translations supplemented by Phonetic Thai/English entries. Five volunteer subjects have been tested on the program at the Institute for Behavioral Research and field tests with an additional ten subjects are being conducted at the SEATO Laboratory in Bangkok, Thailand.

The five subjects who have completed testing at the Institute for Behavioral Research differed widely on initial aptitude test scores, and behavioral records revealed entirely different approaches to the learning situation. All five subjects learned to speak Thai at acceptable levels of proficiency, however, and transcripts of tape-recorded free conversations with natives are available. Achievement tests tapping material presented in the program were administered at selected intervals during the course, and all subjects were evaluated on aural comprehension, phonetic transcription (writing), and pronunciation (tape recordings). Test results were discussed with the subjects and errors on these tests revealed a limited number of recurrent error patterns, suggesting deficiencies in the program which are being corrected in conjunction with content revisions of the course.

Summary and Conclusions.

An autodidactive course in the Thai language for military medical personnel has been developed and preliminary tests completed with laboratory volunteer

subjects. In addition, field tests are in progress at the SEATO Laboratories in Bangkok with the teaching materials emerging from this research. The course in Thai which is now available as a result of this investigative program consists of 1) an audio-visual linear program, 2) a student manual containing additional instructional and evaluative material, and 3) a dictionary containing Thai/English and English/Thai listings. All subjects thus far exposed to the complete 40-section program or even substantial portions of it have learned to speak Thai at acceptable levels of proficiency. Empirically derived data revealed deficiencies in the program, however, and both stylistic and content revisions have been undertaken to remedy such shortcomings.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 086432	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 04 68	A. NEW	U U	NA	GA	A. WORK UNIT

10. CURRENT NUMBER/CODE: 61130011 3AC13001A91C 00 179

10a. PRIOR NUMBER/CODE:

11. TITLE: (U) NOVEL SYNTHESIS OF ORGANOPHOSPHONATES

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012100 ORGANIC CHEMISTRY	04 68	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
	04 68			
	a. NUMBER DADA17 68 C E098	FY 68	0	12
B. CONTRACT	b. TYPE J.C c. AMOUNT $48,504	FY 69	1	36

19. GOVT. LAB/INSTALLATION/ACTIVITY

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

RESP. INDIV.: MERONEY, COL W. B.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: ESSO RSCH + ENGR CO.
ADDRESS: P O BOX 172
LINDEN N J 07036

INVESTIGATORS PRINCIPAL: BROIS, S. J.
ASSOCIATE: BECKER, E.L MD
TEL: NA TYPE: UM

21. TECHNOLOGY UTILIZATION: SYNTHESIS

22. COORDINATION: NA

KEYWORDS: ORGANOPHOSPHONATES, ANTIENZYME ACTIVITY, ALKYLPHOSPHONATES, AMINOALKYLPHOSPHONATES, O-ARYL-O-ALKYL.

24.

(U) TECH OBJECTIVE - TO PREPARE A WIDE SPECTRUM OF O-ARYL-O-ALKYL ALKYLPHOSPHONATE AND O-ARYL-O-ALKYL AMINOALKYLPHOSPHONATE ESTERS WHERE ARYL DESIGNATES P-NITROPHENYL, AND ALKYL SPECIFIES ETHYL, CYCLOPENTYL AND CYCLOHEXYL GROUPS.

(U) APPROACH- THROUGH PROCEDURES AVAILABLE IN THE CHEMICAL LITERATURE, WITH MODIFICATION AS REQUIRED.

(U) PROGRESS - NEW.

26.

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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 179, Novel syntheses of organophosphonates

Investigators.
Principal: Stanley J. Brois, Ph.D.
Associate: Mr. Harvey A. Weiss, B.S.

Description.

The purpose of this project is to synthesize a spectrum of o-aryl-o-alkyl alkylphosphonate and o-aryl-o-alkyl amino-alkylphosphonate esters in which aryl designates p-nitrophenyl and alkyl specifies ethyl, cyclopentyl and cyclohexyl groups. These compounds inhibit in a very characteristic fashion a group of enzymes concerned in certain allergic and non-allergic conditions. As the compounds become available they will be tested in appropriate systems.

Progress.

The project became effective 1 Apr 68. The period between then and 30 Jun 68 was devoted to recruitment of staff, obtaining starting materials and other administrative aspects of the work.

Summary and Conclusions.

None

Publications.

None

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA C84439	CSGRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY AUT	SEC	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 05 68	A. NEW	U	U	NA	GA	A. WORK UNIT

10. a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 180

10. b. PRIOR NUMBER/CODE:

11. TITLE: (U) THE IMPORTANCE OF CHROMIUM IN DISORDERS OF CARBOHYDRATE METABOLISM

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002300 BIOCHEMISTRY 003500 CLINICAL MEDICINE	05 68	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	05 68			
B. CONTRACT	a. NUMBER DADA17-68-C-8119	PRIOR FY 68	0	5
	b. TYPE J.C d. AMOUNT $30,351	CURRENT FY 69	1	25

19. GOVT. LAB/INSTALLATION/ACTIVITY

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

RESP. INDIV.: MERONEY, COL W. F.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: STATE UNIVERSITY OF NEW YORK
ADDRESS: SYRACUSE, N.Y. 13210

INVESTIGATORS PRINCIPAL: DOISY, R. J. PH.D
ASSOCIATE: MERTZ, W. MD
TEL: 315-473-5120 TYPE: DA

21. TECHNOLOGY UTILIZATION: MEDICINE

22. COORDINATION: NA

23. KEYWORDS: ABSORPTION, CHROMIUM, DIABETES, MELLITUS, GLUCOSE TOLERANCE, INSULIN ANTIBODIES, TRACE METALS.

24.

(U) TECH OBJECTIVE - TO STUDY CHROMIUM METABOLISM IN NORMAL, DIABETIC AND ELDERLY HUMAN SUBJECTS AND TO BIOCHEMICALLY DEFINE DEFECTS IN ABSORPTION, HANDLING AND EXCRETION OF CHROMIUM ASSOCIATED WITH IMPAIRED GLUCOSE METABOLISM. TO IDENTIFY THE NATURE OF THE CIRCULATING CHROMIUM COMPLEX WHICH APPEARS IN THE PLASMA IN RESPONSE TO GLUCOSE INGESTION.

(U) APPROACH- ORAL 51-CHROMIUM WILL BE ADMINISTERED AND PLASMA AND URINARY 51-CHROMIUM CONCENTRATIONS WILL BE DETERMINED AT INTERVALS FOR THREE DAYS. THE SAME MEASUREMENTS WILL BE MADE FOLLOWING INTRAVENOUS INJECTION OF 51-CHROMIUM. COMPARISON OF EXPERIMENTAL DATA WILL ALLOW ASSESSMENT OF INTESTINAL ABSORPTION, PLASMA HALF-LIFE AND EXCRETION IN ALL THREE TYPES OF SUBJECTS. STUDIES OF THE NATURE OF CIRCULATING CHROMIUM WILL INITIALLY USE ANIMALS. ELECTROPHORETIC PATTERNS OF CHROMIUM-CONTAINING PLASMA FRACTIONS, BEFORE AND AFTER A GLUCOSE LOAD, WILL BE COMPARED AND THE ELUTED FRACTIONS TESTED FOR BIOLOGICAL ACTIVITY IN THE EPIDIDYMAL FAT PAD ASSAY.

(L) PROGRESS - NEW.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		8R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

37. EST. FUNDS (In thousands)	35.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A013001A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 180, The importance of chromium in disorders of carbohydrate metabolism

Investigators.

Principal: Richard Doisy, Ph.D.
Associate: Walter Mertz, M.D.

Description.

The object of this research is to study chromium metabolism in normal, diabetic, and elderly human subjects and to biochemically define defects in absorption, handling, and excretion of chromium as associated with impaired glucose metabolism. The nature of the circulating chromium complex which appears in the plasma following glucose ingestion will be studied in detail. Oral 51-chromium will be administered and plasma and urinary 51-chromium concentrations will be determined at three-day intervals. Similar measurements will be made following intravenous injection of 51-chromium. Comparison of experimental data will allow the assessment of the importance of intestinal absorption, plasma half-life, and excretion in all three subject groups.

Progress.

None. This project was initiated in May 1968, and is in the preliminary stages at time of this report.

Publications.

None.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 086440	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	D. CHANGE 01 09 67	a. U b. U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61130011 3A013001A91C 00 181

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) DEVELOPMENT OF A MENINGOCOCCAL IMMUNIZING AGENT

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
01C10C MICROBIOLOGY	09 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE c. DATE NA d. AMOUNT NA	FY 68	3	40
		FY 69	3	60

19. GOVT. LAB. INSTALLATION/ACTIVITY

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

RESP. INDIV.: MERONEY, COL W. H.
TEL: 202-576-3551

20. PERFORMING ORGANIZATION

NAME: WALTER REED ARMY INST OF RES
ADDRESS: DIV OF CD AND I
WASHINGTON D C 20012

INVESTIGATORS PRINCIPAL: ARTENSTEIN, M. S. MD
ASSOCIATE
TEL: 202-576-3758 TYPE: DA

21. TECHNOLOGY UTILIZATION: MICROBIOLOGY
PREVENTIVE MEDICINE

22. COORDINATION: NA

23. KEYWORDS: N. MENINGITIDIS, MENINGITIS, POLYSACCHARIDES.

24.

(U) TECH OBJECTIVE - TO ISOLATE, PURIFY AND CHARACTERIZE ANTIGENS FROM MENINGOCOCCI. TO DETERMINE THE PROTECTIVE CAPACITY OF IMMUNOGENIC FRACTIONS.

(U) APPROACH- TO SURVEY MILITARY AND SELECTED CIVILIAN POPULATIONS TO DETERMINE THE PREVALENCE OF THOSE SEROGROUPS OF N. MENINGITIDIS CAUSING DISEASE. POLYSACCHARIDES WILL BE PURIFIED AND CHARACTERIZED BY CHEMICAL AND PHYSICOCHEMICAL METHODS. THE RESPONSE OF ANIMALS AND HUMAN VOLUNTEERS WILL BE MEASURED BY HEMAGGLUTINATION AND BACTERICIDAL ANTIBODY TESTS.

25.

(U) PROGRESS - SEP 67 THRU JUN 68 PURIFIED POLYSACCHARIDES OF GROUP A AND C MENINGOCOCCI HAVE BEEN PRODUCED. THE BASIC UNIT OF A SUBSTANCE IS N-ACETYL MANNOSAMINE PHOSPHATE. THESE PREPARATIONS HAVE A HIGH MOLECULAR WEIGHT AND CONTAIN LESS THAN 1 PERCENT NUCLEIC ACID, PROTEIN AND ENDOTOXIN. NO ANTIBODY RESPONSE WAS FOUND IN MICE, RABBITS, MONKEYS AND CHIMPANZEES. SIX HUMAN VOLUNTEERS DEVELOPED HIGH LEVELS OF BACTERICIDAL AND HEMAGGLUTINATING ANTIBODIES WITHIN TWO WEEKS TO EACH ANTIGEN. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

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Project 3A013001A91C, IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Task 01, In-House Laboratory Independent Research

Work Unit 181, Development of a meningococcal immunizing agent

Investigators.
Principal: CPT Emil C. Gotschlich, MC
Associate: CPT Irving Goldschneider, MC; Malcolm S. Artenstein, M.D.; SP5 Joseph J. Wilczek; and SP5 Robert E. Witker.

Description.

In the past year the Department of Bacteriology undertook preliminary studies to determine whether the group specific polysaccharides of group A and group C meningococci would be suitable antigens for prophylactic immunization against meningococcal disease.

Immunogenicity in human volunteers of these polysaccharides had been tested by Kabat et al. and found to be disappointing. However, a new method allowing the isolation of these polysaccharides in a high molecular weight form encouraged a reexamination of the immunogenic potentials of these materials.

Progress.

Chemical composition of group A and group C polysaccharides.

The chemistry of the group C antigen was described by Watson and Scherp. They showed that the polysaccharide consisted mainly of sialic acid. The chemical composition of the group A antigen has been determined in collaboration with Dr. Teh Yung Liu at Brookhaven National Laboratories. About 90 per cent of the ash free dry weight of the antigen is accounted for by N-acetyl mannosamine phosphate which is also o-acetylated. Furthermore, there is a small amount of another as yet unidentified amino sugar.

Preparation of high molecular weight A and C polysaccharide.

The strains A1 and C11 used for the preparation of these polysaccharides come from the collection of this department and were originally isolated from patients with meningitis. The polysaccharides do not remain cell associated and, therefore, the procedure was designed to isolate them from the whole culture.

Group A or group C meningococci were grown for 16 or more hours in a defined medium containing casamino acids, dextrose, cystine, and salts. One gm Hexadecyl trimethyl ammonium bromide was added per liter of culture. This cationic detergent will precipitate with the acidic polysaccharides, and, of course, other macromolecules such as the nucleic acids. The precipitate was recovered by centrifugation, washed with distilled water

to remove media components, and then triturated with 10% $CaCl_2$. This results in the dissociation of the detergent polysaccharide complex, allowing both to go into solution. The insoluble bacterial debris was collected by centrifugation and was discarded. To the supernate was added ethanol to a final concentration of 25 per cent, resulting in the immediate precipitation of deoxyribonucleic acid and the slower precipitation of ribonucleic acid and other components. The precipitate was removed by centrifugation and the supernate was adjusted to 75-80 per cent alcohol concentration, resulting in the precipitation of the polysaccharide. The detergent and the excess $CaCl_2$ were soluble in alcohol and were removed by washing the precipitated polysaccharide with absolute ethanol. The precipitate was then washed with acetone and ether and vacuum dried.

The dried polysaccharide was dissolved in water and yielded an opalescent solution. The opalescence was sedimented by centrifugation for two to three hours at 100,000 x G and the clear supernate was repeatedly shaken with chloroform to remove contaminating protein.* When this process was completed the polysaccharide was precipitated with ethanol, dissolved in saturated neutral sodium acetate and precipitated with ethanol. This cycle was repeated once and ensures that the polysaccharide was isolated as the sodium salt. The precipitate was dissolved in water, centrifuged for two hours at 100,000 x G to remove a small gelatinous precipitate, and then the supernate was precipitated with three volumes of ethanol, washed with ethanol, and acetone dried. The yields varied, depending mainly on how luxuriant the growth of the organisms were but averaged about 150 mg per 20 liters of culture.

Chemical characterization of high molecular weight A and C antigen.

The antigens isolated by the procedures outlined above were analyzed for their maximum possible contamination with nucleic acid and protein. Nucleic acid was determined by ultraviolet spectroscopy at 260 mμ and protein was determined by the method of Lowry et al. using bovine serum albumin as a standard. The results are presented in Table 1. The preparations indicated with an asterisk were subjected to treatment with copper acetate. It is clear from these results that the copper procedure is not essential. All preparations with two exceptions contained less than one per cent of either nucleic acid or protein. A complete analysis accounting for the dry weight of two representative lots is currently being performed by Dr. Liu.

The average molecular weight of all polysaccharide preparations included in the table was determined by gel filtration over Sephadex G 200. The column had been calibrated with commercial dextran preparations. The effluent of the column was monitored for phosphorus in the

*Some preparations were further purified by adding to them 2/3 volumes of saturated cupric acetate pH 5.0. The solution was allowed to stand and in some instances a small amount of precipitate formed which was removed by high speed centrifugation.

Table 1. Protein and nucleic acid content of meningococcal polysaccharide preparations.

Polysaccharide preparation	Maximum nucleic acid content	Maximum protein content
*Lot A1	0.24%	Not done
Lot A2	0.83%	0.88%
*Lot A3	2.00%	0.79%
Lot A5	0.92%	0.35%
*Lot C2	0.60%	0.62%
*Lot C3	0.85%	1.22%
Lot C4	0.41%	0.31%
*Lot C5	0.18%	0.39%

case of the A polysaccharide and for sialic acid in the case of the C antigen. All preparations eluted as single peaks in the void volume of the column, indicating that they had molecular weights of 200,000 or greater.

Toxicity of high molecular weight A and C antigen.

Four tests for toxicity were employed. Two of these were designed to detect biologically active endotoxin, and the other two were safety tests. Group A polysaccharide A5 and group C polysaccharide C4 and C5 were prepared expressly for human use and the tests were, therefore, performed on the final packaged products, which had passed all sterility tests required by NIH standards. Two and one-half μg amounts of these lots were injected intravenously into three rabbits each and their temperatures were taken at ½, 1, 1½, 2, and 3 hours. Endotoxin was prepared from the same meningococcal strains employing the hot phenol water method of Westphal. One-hundreth of a μg of these endotoxins was injected into six rabbits. The results are listed in Table 2.

It may be seen that none of the rabbits injected with the purified polysaccharides exhibited a febrile response, whereas the rabbits injected with 0.01 μg of endotoxin had easily perceptible pyrogenic responses. By this biological parameter, therefore, the purified group A and group C polysaccharides contained considerably less than one per cent by weight of endotoxin.

These results were confirmed by another biologic test for endotoxin, the inhibition of water intake by mice. White mice bred at Walter Reed weighing ca 20 to 25 gm were weighed and injected intraperitoneally in the afternoon. They were weighed again the next morning and the weight change per mouse was calculated. The results are indicated in Table 3.

Table 3. Weight change of mice inoculated with meningococcal polysaccharides.

Test substance	No. of mice	Weight change per mouse - gm
Saline	20	+ 1.20
C4 100 μg	15	+ 0.92
C5 100 μg	15	+ 0.88
C11 Endotoxin 0.5 μg	15	- 1.98
Saline	20	+ 0.42
A5 100 μg	20	+ 0.31
A1 Endotoxin 0.5 μg	20	- 0.58

These results confirm that the A and C antigens are not significantly contaminated with biologically active endotoxin. Group A polysaccharide

Table 2. Temperature response of rabbits to meningococcal polysaccharides and endotoxins.

Rabbit	Wt. kg	Inoc.	Init.	½ hr	1 hr	1½ hr	2 hr	3 hr
1	2.22	C-4	39.5	39.4	39.3	39.4	39.4	39.3
2	2.12	2.5 µg	39.0	39.0	39.0	39.0	39.0	38.8
3	2.18		39.0	38.8	38.7	38.6	38.7	38.7
6	2.15	C-5	39.0	38.7	38.9	38.8	38.7	38.6
7	2.10	2.5 µg	39.9	39.8	39.8	39.8	39.6	39.5
8	1.92		39.0	39.0	38.8	38.7	38.6	38.5
9	2.43 C11	Endo-toxin	39.8	40.0	40.3	40.4	40.3	40.1
11	2.20	1/100 of µg	39.6	39.9	40.7	40.8	40.5	40.3
12	1.98		39.0	39.1	40.2	40.8	40.3	40.1
13	2.8	A-5 2.5 µg	39.1	39.0	39.1	39.1	39.4	39.3
14	2.65		39.2	39.2	39.3	39.3	39.3	39.2
15	2.03		39.0	39.1	39.3	39.3	39.3	39.3
16	2.38 A1	Endo-toxin	38.7	38.4	39.6	39.9	40.1	39.6
17	2.27	1/100 of µg	38.7	38.7	39.5	39.9	39.8	39.4
18	2.27		38.1	38.0	38.9	39.9	39.7	39.4

lots A2, A3, and C polysaccharide lots C2 and C3 were also assayed by the mouse method just described. In all cases the contamination with endotoxin was less than one per cent.

Two tests for general toxicity were employed. The first is the standard safety required by the National Institutes of Health for biological products and consisted of the intraperitoneal injection of 500 μg of polysaccharide into guinea pigs. This represents ten human doses. This test was done on lots C4, C5, and A5. All guinea pigs gained weight over a one week period and no febrile responses were noted.

The other test for general toxicity consisted of injecting 14-16 gm white mice with 100 μg of polysaccharide intraperitoneally and comparing their weight gain to a control group for a one week period. The weight gain of the mice injected with polysaccharide was no different from the saline controls.

Table 4. Toxicity of polysaccharides in growing mice.

Expt.	Treatment	No. of mice	Weight gain/ mouse (gm)
1	Saline	20	+ 8.32
	C4 100 μg	20	+ 8.30
	C5 100 μg	20	+ 8.12
2	Saline	25	+ 9.00
	A5 100 μg	20	+ 7.60
3	Saline	10	+ 6.1
	A2 100 μg	10	+ 6.0
	A3 100 μg	10	+ 6.5
	C2 100 μg	10	+ 6.1
	C3 100 μg	10	+ 5.9

Immunogenicity of high molecular weight A and C antigen in laboratory animals.

It has been repeatedly shown that highly purified polysaccharides are excellent antigens in human beings but are poorly antigenic in the common laboratory animals. The best quantitative illustration of this point is a comparison of the average human antibody response to pneumococcal polysaccharide (ca 50 μg of antibody protein/ml of serum) and the response of the mouse (ca 7.5 μg of antibody per ml). The rabbit and the guinea pig are reputedly even poorer responders. The antigenicity of pneumococcal

polysaccharides in mice has been studied extensively in the past since a ready measure for antibody production was available by challenging the mice with living pneumococci of the appropriate type. This work in mice has delineated the optimum dose of polysaccharide to be used for immunization (0.1 to 1.0 μg) and the moment of maximum immunity (one week following immunization).

Work carried on in this laboratory in the past two years has indicated that inbred mice of strain C57Bl/6 MAI are ideal for mucin enhanced meningococcal infection. The majority of the mice of this strain die when injected with ten or more meningococci suspended in five per cent hog gastric mucin, and these mice can be passively protected against fatal meningococcal infection by prior injection of antiserum.

This model, therefore, was used to determine the antigenicity of the A and C polysaccharide. Several experiments were performed, all indicating that no protective effect whatsoever could be ascribed to immunization with A or C polysaccharides. One of the experiments is described in Table 5.

Table 5. Challenge of mice following immunization with meningococcal polysaccharides.

Immunization	Challenge	No. of mice	Survivors
A5 0.5 μg	100 A1	20	2
A5 0.1 μg		20	4
A5 0.05 μg		20	3
Controls		20	4
C5 0.5 μg	100 C11	20	3
C5 0.1 μg		20	2
C5 0.05 μg		20	4
Controls		20	3

Mice were injected I.P. with antigen with a range of dosage designed to encompass the optimum dose. One week later the animals were challenged by intraperitoneal injection of approximately 100 meningococci of the appropriate type, suspended in mucin. This unusually low dose was chosen to maximize the opportunity of observing active immunity.

It should, however, be borne in mind that the two meningococcal polysaccharides under consideration may be just as antigenic as the pneumococcal polysaccharides but that the mucin challenge overwhelms the immune defenses of the mouse.

The immunogenicity of the meningococcal polysaccharides was also tested in a single experiment in rabbits. One hundred μg of group A polysaccharide was emulsified in complete Freund's adjuvant and administered to a rabbit. The same procedure was carried out with 100 μg of C polysaccharide. This dose and method was employed since it has been successful in the immunization of rabbits with synthetic polypeptides which in many instances are poor antigens. The rabbits were bled one week and five weeks following immunization and the antibody response measured by passive hemagglutination. No increase in titer was noted.

Three species of primates were also immunized and bled at appropriate intervals and their sera tested by passive hemagglutination. A total of 13 rhesus monkeys were injected with A and C polysaccharide. Three monkeys were injected with 25 μg of A antigen and three monkeys with 25 μg of C antigen. Three weeks later these animals were reinjected with 20 μg of the same antigen they had received in an attempt to test the effect of multiple doses. Furthermore, the same animals were injected with 2 μg of the opposite antigen. Six further animals were injected with 2 μg of antigen, three animals with A and three animals with C. One animal who was larger, weighing about 15 lbs, was injected subcutaneously with 50 μg of A substance absorbed into alum. None of these animals exhibited any significant increase in their hemagglutination titers.

Four chimpanzees were immunized with 50 μg of A substance and four chimpanzees with 50 μg of C substance; half of the animals were intact, the rest splenectomized. No changes in hemagglutination titers were observed.

Five gibbons located in Bangkok, Thailand were injected with 25 μg of antigen intradermally. No change in titers was observed in four of these animals. However, one gibbon produced a definite immune reaction. Its titer rose from 1/8 to 1/128 within a week and declined to 1/64 over the next two weeks. It should be noted that only a single animal showed a response and that it conceivably could have arisen from other causes, such as inapparent infection either with a group C meningococcus or another species of organism with a cross reactive antigen.

In light of the findings to be described in the following section on humans, it is very odd that the monkeys and apes had serological behavior so strikingly different from the human being.

Immunogenicity of high molecular weight group A and group C antigen in human volunteers.

The group specific polysaccharides were injected intradermally into the forearm in doses of 50 μg into adult human volunteers. Six subjects received the C vaccine and five of these also the A vaccine. Their sera were tested by several parameters. The subjects were also throat cultured in the course of the study. All cultures were negative except for subject I.G. who had just acquired a group B meningococcus. The results are shown in Tables 6 and 7.

Table 6. Reciprocal hemagglutination titers with cells sensitized with group C polysaccharide.

	Weeks following immunization with C antigen								
Subjects	0	1	2	3	4	5	6	10	20
MSA	2	64	256	256	256		256		
WCS	2	32	256	256	256				
IG	8	32	128	128	128		128		
ECG	2	128	256	128		128		128	128
JS	2	64	64	64	64		64		
JW	32	64	128	128	128		128		

Table 7. Reciprocal hemagglutination titers with cells sensitized with group A polysaccharide.

	Weeks following immunization with A antigen							
Subjects	0	1	2	3	4	7	12	22
MSA	8	128	1024		1024			
IG	32	128	512		512			
ECG	8		512	512	512	512	512	256
JS	16	64	128		128			
JW	16	512	512		512			

Every vaccinated subject responded to a greater or lesser degree. The three week sera were tested for the presence of precipitating antibody employing the ring test. All vaccinated subjects but JS had detectable precipitating antibody.

The antibody response of these subjects was also tested, employing the antibody complement mediated bactericidal reaction of serum. The sera were diluted serially in a human serum which was known not to contain bactericidal antibodies. This serum served as the complement source. Standard strains of meningococci were incubated for 30 minutes in each dilution and the survivors estimated by plate counts. Strains A1 and 121 Misc were group A, and C11 and 107 VI were group C strains. The second column indicates the date of immunization.

Table 8. Serum bactericidal antibody in six humans following immunization.

Subject		Immunogen		Bactericidal titer A-1	121 Misc	C11	107 VI
MSA	5 Apr	C5	5 Apr	< 4	< 4	16	16
	12 Apr			< 4		128	512
	19 Apr	A5	19 Apr	< 4	< 4	2048	1024
	26 Apr			32		2048	
	3 May			128	256	2048	
	17 May						
WCB	1 Apr	C5	5 Apr	4	4	8	8
	12 Apr			4		16	64
	18 Apr			4	4	128	256
	26 Apr			4			
	3 May			4	8	128	256
	17 May			4			
IG	2 Apr	C5	5 Apr	32	32	16	16
	12 Apr			32	16	32	64
	19 Apr	A5	19 Apr		16	128	128
	26 Apr			128			
	3 May			64	256		
	17 May			64		256	
JS	2 Apr	C5	5 Apr	8	8	4	4
	12 Apr			8		64	64
	19 Apr	A5	19 Apr	8	16	256	256
	26 Apr			32			
	3 May			32	128		
	17 May			32		512	256
JW	2 Apr	C5	2 Apr	8	8	128	
	12 Apr			8		256	
	19 Apr	A5	19 Apr	8	8	1024	
	26 Apr			128		1024	
	3 May			256	256	1024	
	17 May			256		1024	
ECG	30 Nov	A2	30 Nov	4	8	8	< 4
	7 Dec			8			
	11 Dec			64			
	14 Dec	C2	14 Dec	64		16	
	21 Dec			64	128	256	
	28 Dec			64		256	
	5 Jan			64		256	
	18 Jan			64		256	
	21 Feb			64		256	512
	5 May					256	256

The bactericidal activity of the serum of subject ECG was studied more extensively and is shown in Table 9.

Table 9. Bactericidal antibody following immunization of one adult human.

Meningococcal strain	Sero-group	Bactericidal titer ECG (30 Nov)	ECG (14 Dec)	ECG (21 Feb)
A-1	A	4	-	64
120-Misc	A	8	-	64
121-Misc	A	8	-	128
C-11	C	8		256
182-I	C	8		256
91-II	C	8		128
176-III	C	8		256
158-IV	C	8		128
140-V	C	8		128
107-VI	C	<4		512
85-III	B	8	8	8
122-Misc	135	8	8	8
166-IV	B	8	8	8
169-III	B	4	4	4
153-I	B	8	8	8

This data illustrates well the specificity of the immune reaction obtained in response to these vaccines. No increases in bactericidal antibody to group B or group 135 were observed in response to immunization.

Subject I.G., as mentioned before, was a B carrier at the time of the experiments. The hemagglutination responses of this subject are definitely due to the vaccination since there is ample data to show that neither the group B carrier state nor group B meningococcemia gives rise to hemagglutinating antibodies to A or C substance. However, the increase in bactericidal response could well be a composite of the

natural infection and the artificial immunization. Studies designed to give a semiquantitative estimation of the different classes of antibodies produced in response to these vaccines have been carried out. The experiment consists of allowing either group A or group C meningococci to dry on microscope slides. The sera to be tested are then dropped in serial two-fold dilutions onto the organisms and the antibodies allowed to react for 20 min. The slides are then washed and stained with commercial fluorescein tagged antisera obtained from rabbits immunized with isolated heavy chains of IgG, IgM and IgA. The slides are washed and graded by fluorescence microscopy. Table 10 indicates the results obtained. The data is not yet complete but indicates that all subjects produced all three classes of immunoglobulins and that there was considerable individual variation.

Having obtained these encouraging results, it was decided to test these substances in a greater number of people. The experiment was designed to test the immunogenicity and the lack of toxicity in a greater number of people and also to examine whether immunization with the group C polysaccharide would decrease the incidence of group C nasopharyngeal carrier state. This question, aside from interesting theoretical implications, is of practical importance because it would be difficult to design any larger protective trial without this information. Three companies in first week of BCT at Fort Dix were chosen for study. There has been an unusually high incidence of group C meningococci carrier state and disease in the past year. The recruits of these companies were bled, throat cultures taken and then informed consent was obtained. The volunteers were asked to fall-in in their usual platoons and, wherever possible, equal numbers of men in each platoon were immunized. This was to ensure a random mixture of the control and the immunized population. The vaccinated subjects were observed for 48 hrs and no instance of systemic toxicity was observed. All subjects had a local reaction consisting of 3-7 cm of erythema, slight induration, and tenderness. This reaction faded in 48 hrs.

One hundred and forty-five recruits were injected with C polysaccharide and 52 with the A polysaccharide, as described below in Table 11.

Table 11. Number of recruits vaccinated.

Company	C vaccinated	A vaccinated
B-6-3	50	16
E-5-3	45	0
E-2-3	50	36

The reason for the discrepancy among the companies was that only 45 recruits in Company E-5-3 volunteered.

Table 10. Identification of specific immunoglobulin antibodies following immunization of five humans.

Date	Immunogen	MSA		WCB		IG		JS		JW	
		A-1	C-11	A-1	C-11	A-1	C-11	A-1	C-11	A-1	C-11
						Immunofluorescence Titer - IgG					
5 Apr	C5	<4	4	4	4	>8	>8	8	4	16	4
12 Apr		<4	64	4	32	>64	64	8	64	16	32
19 Apr	A5	<4	256	4	128	256	128	16	64	16	128
26 Apr			256	4	256			32			
3 May			256					64			
17 May								64		256	
						Immunofluorescence Titer - IgM					
5 Apr	C5	<4	<4	8	4	>4	8	<4	4	8	16
12 Apr		<4	16	8	32	16	32	<4	32	8	64
19 Apr	A5	<4	32	8	128	>64	64	<4	32	8	64
26 Apr			64	8	128			32			
3 May			64					32			
17 May								32		256	
						Immunofluorescence Titer - IgA					
5 Apr	C5	2	<4	8	8	>4	>4	<4	4	8	4
12 Apr		2	32	8	8	>16	>16	<4	16	8	16
19 Apr	A5	2	256	8	128	>64	>64	<4	32	8	128
26 Apr			256	8	128			64			
3 May			256					128			
17 May								64		256	

All the recruits were bled and cultured two weeks following vaccination in their third week of training. They were again cultured two weeks later in their fifth week of training, and it is planned to obtain the final bleeding and throat culture in their seventh week of training. The serological data will not be available until the termination of the study. However, the cultural data for the first two cultures are completed and are reported in Table 12. Only the C carrier state is recorded and the third company is omitted because the C carrier rate was negligible.

Table 12. Effect of immunization of meningococcal carrier rates.

		Group C meningococcal carriers among		
Company	Culture	C immunized	A immunized	Controls
B-6-3	0	3 (6%)	0	5 (2.7%)
	14 d	1 (2%)	1 (6.2%)	20 (11.2%)
E-2-3	0	2 (4%)	1 (2.8%)	6 (5%)
	14 d	5 (10%)	13 (36%)	37 (30.3%)

At the beginning of the study the C carrier rate was low and randomly distributed. However, two weeks later there was considerable increase in the C carrier rate, particularly in Company E-2-3. The C carrier rate among the controls and the recruits immunized with A polysaccharide is similar. However, the C carrier rate among the C vaccinated recruits is distinctly lower. This difference was examined statistically by the method of Chi square and found to be significant at better than the one per cent level. Company B-6-3 shows the same trend and the difference between the controls and the vaccinated group is significant at the five per cent level.

It is, therefore, probable that immunization with the C polysaccharide has an effect on reducing the acquisition of meningococci in the nasopharynx.

Summary and Conclusions.

A reliable method was developed for the isolation of group A and group C meningococcal polysaccharide in a high molecular weight form and in high purity. Animal experiments indicated that these materials were not toxic. These materials were found to be poor antigens in mice, rabbits, rhesus monkeys, gibbons, and chimpanzees. However, both polysaccharides proved to be good antigens in human volunteers. They have been administered to over 200 people and no toxic reactions have been observed. There is evidence that immunization with the group C polysaccharide will prevent the acquisition of the group C meningococcal nasopharyngeal carrier state.

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
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		CURRENT FY 69	9	140

19. GOVT. LAB/INSTALLATION/ACTIVITY
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TEL: 202-576-3528 TYPE: DA

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KEYWORDS: PROTEINS, PROTEIN SYNTHESIS, FATTY ACIDS, ENZYMES, BACTERIAL METABOLISM, RIBONUCLEIC ACIDS, ENTEROBACTERIACEAE.

23.

(U) TECH OBJECTIVE - BASIC BIOCHEMICAL RESPONSES TO INJURY AND IN DISEASE WILL BE STUDIED. POTENTIAL MEANS OF CONTROLLING INFECTIOUS AGENTS BY BIOCHEMICAL REGULATORY MECHANISMS WILL BE INVESTIGATED.

(U) APPROACH- THE MODERN TECHNIQUES AND ANALYTICAL APPROACHES AVAILABLE TO RESEARCH BIOCHEMISTRY WILL BE USED TO EVALUATE RESPONSES AND TO STUDY THE BASIC CAUSES FOR DISEASE PRODUCTION BY INFECTIOUS AGENTS.

24.

(U) PROGRESS - JUL 67 THRU JUN 68 PROGRESS IN DEFINING THE NUCLEOTIDE SEQUENCE OF TYROSINE-TRANSFER RIBONUCLEIC ACID (T-RNA) FROM E. COLI ALLOWS A FORMAL PROPOSAL OF PRIMARY AND SECONDARY STRUCTURE OF T-RNA. LARGE-SCALE ISOLATION AND EXTENSIVE PURIFICATION OF E. COLI T-RNA, IN PROGRESS, WILL PROVIDE MATERIAL FOR FINAL IDENTIFICATION OF NUCLEO-TIDE BASES. A PROCEDURE FOR DETERMINING THERMAL STABILITY OF REASSOCIATED DNA ON HY-DROXYAPATITE PROVIDES A METHOD WITH SENSIVITY AND REPRODUCIBILITY IDENTICAL TO THAT OF COLUMN ASSAY WITH A FIVE-FOLD INCREASE IN CAPACITY. EXPERIMENTS TO DETERMINE HOMOLOGIES IN DEOXYRIBONUCLEOTIDE SEQUENCES BETWEEN THE DNA OF BACILLUS SUBTILIS AND THAT OF SEVERAL BACTERIOPHAGES ARE PROVIDING INSIGHT INTO CONSERVATION OR DIVERGENCE OF SPECIFIC GENETIC REGIONS WITHIN THE DEOXYRIBONUCLEIC ACIDS OF RELATED ORGANISMS. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

26.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In Thousands)	36.

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Project 3A014501B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 070, Biochemical activity in health and disease

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Description.

This project provides basic research support for biochemical studies of the various factors which contribute to the processes of disease. A principal, and most rewarding, avenue of approach has been through the study of the mechanisms of protein synthesis by a variety of pathogens. These studies have included the various genetic variations which may be instituted under both artificial and natural conditions and the detailed study of the nucleic acids which are characteristic of the species under investigation.

Progress.

Mechanism of protein synthesis. The course of amino acids from the free form in cytoplasm to correct placement in a growing peptide chain involves a series of highly specific and precise reactions. The molecule which is crucially involved in this series, transfer RNA, must be very special, for it must recognize sites on specific enzymes, ribosomes, messenger RNA chains, and one amino acid. It is believed that the exact linear sequence of nucleotides in certain areas in the molecule and/or the resulting three dimensional configuration are the determinants in its ability to function correctly. Virtually nothing is known about the mechanism of interaction between nucleic acids and proteins and its essential function in DNA, RNA, and protein synthesis. Recently it was shown in this laboratory that the amino acid charging capability of $tRNA_{tyr}$ from yeast and E. coli was species specific, that is, in heterologous systems the corresponding enzymes which activate and attach the proper amino acid in position on the tRNA were not recognized by tRNA from the other species. This finding suggested that the binding sites were structurally different on either the enzymes or the tRNAs or both. Since the nucleotide sequence of yeast $tRNA_{tyr}$ was already known, it became imperative to elucidate the chemical structure of E. coli $tRNA_{tyr}$ to attempt to locate the enzyme-binding site.

$tRNA_{tyr}$ from E. coli was purified using countercurrent distribution procedures developed in this laboratory. The highly purified $tRNA_{tyr}$ was subjected to selective fragmentation by highly specific endoribonuclease digestion under various controlled conditions. The resulting fragments were separated by column chromatography and were further sequentially degraded by enzymatic and chemical hydrolysis. Base compositions of these pieces were then determined by chromatographic, spectral, and radioisotopic analyses. Sequence positions of the nucleotides were determined by juggling the overlapping fragments.

Elucidation of the nucleotide sequence of E. coli $tRNA_{tyr}$ has been completed. Striking similarities and dissimilarities to the structure of yeast $tRNA_{tyr}$ can be noted:

(1) A cloverleaf secondary structure as proposed by Holley et al. can be derived for E. coli $tRNA_{tyr}$ from its nucleotide sequence.

(2) E. coli $tRNA_{tyr}$ contains 87 nucleotide bases, whereas yeast $tRNA_{tyr}$ contains 78 bases; the extra bases in the former being located in the "lump" between the anticodon (messenger RNA-recognizing group) and the TψCG loops. Otherwise, the number of bases in the basic loops and hydrogen-bonded areas are the same in both species, and 48 corresponding base positions on the molecules are the same.

(3) The amino acid acceptor end (3' end) of both E. coli and yeast $tRNA_{tyr}$ has the sequence -A-C-C-AOH. The 5' end of the E. coli tRNA is pGp in contrast to pCp of the yeast tRNA.

(4) The nucleotide sequence in the anticodon and -T-ψ-C-G- loops is extremely similar in both tRNA's. The anticodon of both contains the same major bases although they differ in base modification.

(5) The bases on the 5' end of the bonded region of the stem are mainly pyrimidines in E. coli $tRNA_{tyr}$ with corresponding purines on the 3' end; in yeast the case is just reversed.

(6) Dihydrouridine is absent from E. coli $tRNA_{tyr}$, whereas yeast $tRNA_{tyr}$ contains 6 molecules and all other determined yeast tRNA's contain 2-4 molecules.

(7) E. coli has only one methylated base other than the thymidine common to all known tRNA's. Yeast $tRNA_{tyr}$ contains 6 methylated bases; the other yeast tRNA's contain 2-8 molecules.

(8) A sulfur group is believed to modify 3 bases in E. coli $tRNA_{tyr}$, one immediately adjacent to the anticodon; there are no thio derivatives in yeast $tRNA_{tyr}$, nor have any been determined in other yeast tRNA's.

The main differences between $tRNA_{tyr}$ from E. coli and yeast lie in the dihydrouridine-containing loop, the 5'-3' stem and the "lump," with the lack of dihydrouridine and methyl groups, and the presence of sulfur in E. coli. Any or any combination of these differences may contribute to conferring the species specificity in addition to the general enzyme-recognition and ribosome-binding abilities of the molecule. This information is useful in attempting to understand the essential phenomenon of intermolecular action in the normal and aberrant state and the evolutionary development of species in the phylogenetic scale. It appears that further investigations can be performed with tRNA specifically modified in certain regions or positions in order to locate functional sites by altered activity. Large scale purification of $tRNA_{tyr}$ for this purpose is now in progress.

b. Biochemical host-parasite relationships. As a model for studying the biochemical aspects of host-parasite relationships, experiments have been designed to look for reassociation of nucleotide sequences between the deoxyribonucleic acid (DNA) of Bacillus subtilis and that of several bacterial viruses for this organism. The procedure involves the measurement of reassociation of single-stranded DNA which has been formed by heat or alkali denaturation of the double helix. The assay is based on the ability of hydroxyapatite to bind double-stranded, but not single-stranded, DNA. An alternative procedure involves the binding of DNA fragments to DNA immobilized in agar. The amount of reassociation between heterologous DNA preparations is a measure of the amount of related nucleotide sequences in each of the genomes.

Work within the past year has been directed toward (1) the isolation and characterization of new phages from soil and sewage in an attempt to obtain both virulent and temperate types for comparison and (2) preparation of radio-labeled DNA from both host strains and phage particles.

(1) Isolation and characterization of B. subtilis phages. Two new phages for B. subtilis have been isolated and selected for further study. Phages antigenically different from those previously characterized (Yehle and Doi, J. Virology, 1, 935, 1967) were obtained by the addition of specific phage antisera to the isolation medium. They have been further differentiated by their host range specificity, plaque morphology, and the physical properties of their DNA preparations. Equipment is presently being ordered to permit the determination of thermal denaturation profiles of the DNA of these phages. One of these is a virulent phage while the other appears to be temperate.

(2) Preparation of radiolabeled host and phage DNA. Experiments are now in progress to prepare isotopically labeled DNA from Bacillus subtilis strains and several of its phages. Preparations of high specific activity (about 20,000 cpm/μg) are necessary to obtain the proper sensitivity in the hydroxyapatite system. Thymine-requiring mutants of the host strains have been obtained for use in labeling host and phage DNA preparations with ^{14}C-thymine. Stocks of unlabeled DNA are also being prepared for use in the assay systems.

c. Isolation and characterization of nucleic acids. Techniques for the isolation and characterization of nucleic acids from bacterial and animal cells are being applied to the study of the nucleic acids of Plasmodium knowlesi. The problem involves the separation of parasites from other DNA-containing cells in the blood of infected monkeys. Chemical and physical characteristics of the purified parasite DNA are being determined to elucidate methods for antimalarial therapy as well as the mechanism of action of compounds already proven effective in malaria prophylaxis.

Isolation of parasites from infected monkey red blood cells has been concerned with the isolation of parasites by rupture of host cell cytoplasmic membranes, leaving the intracellular parasites intact. This has been accomplished by manipulation of the osmotic conditions surrounding the cells. The efficiency of the procedure was monitored by direct microscopic observation of stained preparations.

Isolation and characterization of DNA from Plasmodium knowlesi. DNA was isolated from parasites provided by CPT Cook and whole monkey blood supplied by CPT Barnes by either phenol extraction (Davison and Freifelder, J. Mol. Biol., 5, 643, 1962), or by the chloroform method of Marmur (J. Mol. Biol., 3, 208, 1961). Aliquots were banded at equilibrium in CsCl density gradients using deuterated E. coli DNA as a reference. The density of P. knowlesi DNA in CsCl is 1.697 g/cc which, from the relationship shown by Schildkraut et al. (J. Mol. Biol., 4, 430, 1962), corresponds to a base composition of 37.7% guanine plus cytosine (GC). The density of normal monkey blood DNA is 1.699 g/cc corresponding to a base composition of 39.8% GS. Equipment is now being ordered for the determination of thermal denaturation profiles for each of the DNA preparations.

d. Studies of interspecies DNA complexes. Recent techniques, including the binding of deoxyribonucleic acid (DNA) fragments to DNA immobilized in agar (Bolton and McCarthy, Proc. Nat'l Acad. Sci., U.S. 48: 1390, 1962), and the ability of calcium phosphate (hydroxyapatite) to discriminate between single- and double-stranded DNA (Miyazawa and Thomas, J. Mol. Biol. 11: 223, 1965), allow one to react single-stranded DNAs from different organisms and specifically look at the reassociated nucleotide sequences. Our efforts during the past year entailed the use of these techniques: (1) to investigate host-parasite relationships using bacteriophage bacterium interaction as a model host-parasite system; (2) to obtain information about relatedness among pathogenic and non-pathogenic bacteria at the molecular level; (3) to specifically investigate and perhaps isolate specific regions of the bacterial chromosome; (4) to increase the efficiency of the hydroxyapatite method for fractionating DNA.

(1) Conservation of temperate bacteriophage DNA (in collaboration with Dean B. Cowie, Carnegie Institution of Washington, Department of Terrestrial Magnetism, and Stanley Falkow, Georgetown University School of Medicine, Department of Microbiology). The well-established genetics of temperate

coliphages make them an ideal system in which to study evolutionary relationships. Relatedness among temperate coliphages was investigated using an extension of the DNA-agar method. The thermal stability of DNA fragments bound to agar-trapped DNA was determined by eluting the bound fragments in a series of washes at increasing 2.5°C temperature increments. In all cases, DNA from phages tested (including λ, 434, Ø80 and P_{22}) contained significant sequences that were able to reassociate with λ DNA (20% for Ø80 and P_{22} to at least 50% for 434). Thermal elution profiles obtained from studies of reactions among phage DNAs show that the interspecies phage DNA reassociation products appear virtually identical to those obtained with DNA from the same species. Similar experiments carried out using phage-Escherichia coli DNA show that approximately 40% of the DNA from λ, 434 or 434 hy can reassociate with E. coli DNA. The stability of phage-E. coli DNA duplexes is similar to that observed in studies involving reactions in which both the labeled DNA fragments and the DNA trapped in the agar were from E. coli. There are, however, some nucleotide sequences which elute at low temperatures in the phage-host reactions.

These observations have been interpreted to indicate extensive conservation among the nucleotide sequences of temperate enterophage DNAs which can reassociate and among many of the nucleotide sequences held in common by the DNA of these phages and their host bacterial cells.

Analogous phage-phage and phage-host studies have been initiated in strains of Bacillus subtilis and bacteriophages which attack these strains. These studies are in collaboration with CPT Clifford Yehle.

(2) Thermal stability of interspecies enterobacterial DNA duplexes (in collaboration with Dean B. Cowie and Stanley Falkow). The amount of interspecies DNA duplex formation between E. coli and Salmonella typhimurium decreases when the incubation temperature is increased. This observation prompted a study of thermal stability in interspecies enterobacterial DNA duplexes. Radiolabeled E. coli DNA fragments were allowed to reassociate at 60°C or 75°C with a large excess of unlabeled DNA fragments from various enterobacteria. The reassociated DNA was adsorbed to a hydroxyapatite column (under conditions where single-stranded DNA was not adsorbed to the column) and subjected to a series of washes at increasing temperatures. As the bound DNA was thermally denatured it was eluted from the column and the T_m (that temperature at which 50% of the DNA is denatured and eluted from the column) of the interspecies DNA duplex was determined. The stability of E. coli-Shigella flexneri duplexes at either temperature was identical and similar to that of reassociated E. coli DNA duplexes. The degree of interspecies duplex formation was minimally affected by increasing the incubation temperature (86% at 60°C to 80% at 75°C). Interspecies DNA duplexes formed at 60°C between E. coli and Aerobacter aerogenes, S. typhimurium or Proteus mirabilis had a T_m 10-13°C below that of E. coli. At 75°C the formation of these interspecies DNA duplexes was markedly decreased (4-8 fold), however, the stability of the DNA able to reassociate at 75°C approximated that of reassociated E. coli DNA. The relatedness between Sh. flexneri and E. coli,

as judged by the ability of their DNAs to reassociate and the thermal stability of the resulting DNA duplex, is indicative of little evolutionary divergence in these organisms. The other enterobacteria tested have diverged to a point where not more than 40% of their DNA can reanneal with E. coli DNA at 60°C and less than 10% can react at 75°C. Relatedness among the Enterobacteriaceae continues to be of major interest to our laboratory. In addition, we have started to investigate the stability of interspecies Neisseria DNA duplexes. These studies are being carried out jointly in our laboratory and in the laboratory of David Kingsbury at the National Naval Medical Center. Preliminary results indicate that both interspecies duplexes with high and low stability are present among species of Neisseria.

(3) Identification of specific regions of the bacterial chromosome (in collaboration with Stanley Falkow). Some 7-10% reassociation occurs between the DNAs of E. coli and Salmonella typhosa at 75°C; and less than 1% reassociation occurs between the DNAs of E. coli and Proteus mirabilis at 75°C. These are ideal systems in which to detect regions of E. coli DNA due to this low background binding at 75°C. A mutant of P. mirabilis containing an f-lac episome from E. coli and three S. typhosa diploid strains carrying differing amounts of E. coli DNA were tested. In all four cases it was possible to detect the E. coli DNA by increased binding of the Proteus f-lac and the Salmonella diploid strains to E. coli DNA at both 60°C and 75°C. It was also evident that the more E. coli material present in these strains, the more stable was their reassociation product with E. coli DNA. It should be possible to more intensively study specific regions of the chromosome and to possibly isolate these regions by appropriate DNA reassociation experiments with these and other mutant and diploid strains.

(4) A batch procedure for thermal elution of DNA from hydroxyapatite (in collaboration with Adrian V. Rake, Carnegie Institution of Washington, Department of Terrestrial Magnetism). The hydroxyapatite method for the fractionation of single- and double-stranded DNA has several advantages over other techniques. Despite these advantages the hydroxyapatite method is time-consuming and only one or two columns may be handled simultaneously, whereas ten or more samples may be simultaneously processed using other methods. We have been working on a batch procedure for the thermal elution of DNA from hydroxyapatite. Preliminary results indicate that this method is faster and cheaper to use than the column procedure and that it allows one to handle up to ten samples simultaneously. In this procedure the hydroxyapatite fractionations are carried out in centrifuge tubes. The washing solution is removed by rapidly sedimenting the hydroxyapatite-buffer mixture in a heated centrifuge, pouring off the buffer and rapidly resuspending the hydroxyapatite pellet in fresh, preheated buffer.

Summary and Conclusions.

Previous studies have suggested that some transfer RNAs from different species are structurally different. The primary nucleotide sequence of tyrosine E. coli tRNA has been determined. The differences of structure

between this and yeast $tRNA_{tyr}$ suggest a chemical basis for their interaction with proteins and nucleic acids and their evolutionary development.

Recent observations of interspecies DNA reassociation among the temperate bacteriophages for E. coli bear repeating with another phage-host system. Several temperate phages for B. subtilis have been reported and one lambda-like phage, SPO2, in particular, has been described by Romig (Symposium on Entry of Foreign Nucleic Acids, Fort Detrick, Maryland, April 4-5, 1968). Attempts are being made to obtain similar phages which are truly temperate for B. subtilis in that the phage genome integrates with the host chromosome as in the case of lambda phage.

DNA has been isolated from malarial parasite Plasmodium knowlesi and has been compared with that from normal whole monkey blood. Normal distribution patterns of the parasite DNA were found in CsCl density gradients. These preliminary studies indicate that the parasite DNA has been obtained reasonably free of contaminating host animal DNA. They also show that the density and base composition of the parasite DNA is within the range of values reported for mammalian DNA.

Interspecies DNA reassociation studies have shown that temperate enterophages are related to one another in all cases where tested. The temperate enterophages are all also related to E. coli. The most significant aspect of the relatedness between the DNAs of temperate phages and between temperate phage and E. coli DNA is that the related nucleotide sequences appear to be just as stable as intraspecies DNA duplexes; therefore, these related sequences are assumed to be extensively conserved. Relatedness is not evident between the DNA of virulent bacteriophages or between the DNA of these phages and E. coli DNA. Closely related bacteria have lost very little common DNA and the related sequences among them have not extensively diverged. The less closely related enterobacteria show 40% or less DNA reassociation; therefore, the majority of nucleotide sequences in these organisms have diverged to a point where they can no longer reassociate with one another. The significantly decreased stability evident in these interspecies DNA duplexes indicates that these sequences have also apparently diverged extensively. The batch hydroxyapatite method currently being tested in our laboratory should greatly increase the number of samples that can be processed.

Publications.

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21. TECHNOLOGY UTILIZATION	22. COORDINATION
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24.

(U) TECH OBJECTIVE - TO EVALUATE NEWLY REPORTED TECHNIQUES FOR DIAGNOSTIC ASSISTANCE TO THE PHYSICIAN AND TO DEVELOP ADDITIONAL CONCEPTS TO BE USED FOR DIFFERENTIAL DIAGNOSIS OF DISEASE.

(U) APPROACH- THOROUGH EVALUATION OF NEWLY REPORTED DIAGNOSTIC BIOCHEMISTRY WILL BE ACCOMPLISHED AND IN ESPECIALLY PROMISING DEVELOPMENTS THE TECHNIQUES WILL BE APPLIED TO PROGRAMS WITHIN THE ARMY MEDICAL SERVICE. WHERE SOME TECHNIQUES ARE LACKING, RESEARCH WILL BE DONE TO DEVELOP NEW PROCESSES FOR DIAGNOSTIC EVALUATIONS

(U) PROGRESS - JUL 67 THRU JUN 68 INVESTIGATIONS OF BIOCHEMICAL CHANGES IN CEREBROSPINAL FLUID (CSF) RESULTING FROM NEUROLOGIC DISEASES HAS CONTINUED. QUANTITATIVE CSF PROTEIN ELECTROPHORESIS STUDIES OF PATIENTS WITH DEMYELINATING DISEASES HAVE SHOWN MARKED ELEVATIONS IN TOTAL GAMMA GLOBULIN FRACTIONS AND SIGNIFICANT ELEVATION (20-25) OF THE LDH-3 ISOENZYME IN PATIENTS WITH MYELIN LESIONS. A SYSTEM OF AGAR GEL ELECTROPHORESIS HAS BEEN DEVELOPED TO DETERMINE PROTEINS, ISOENZYMES, AND LIPOPROTEINS. STUDIES ON STRESS HAVE SHOWN, A(FREE CHOLESTEROL IS REDUCED BY ONE-HALF IN RATS SWIMMING 4 HOURS DAILY FOR 4 WEEKS, B) EXERCISE INCREASED PLASMA FATTY ACIDS WITH THE CHANGE DIMINISHING WITH TRAINING, C) EXERCISE DECREASED PLASMA TRIGLYCERIDES, D) ISOENZYME RESPONSES WERE DIMINISHED WITH TRAINING AND EXERCISE. FURTHER WORK IS CONTINUING. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 071, Biochemical variations in abnormal health states

Investigators.

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Description.

This project provides an opportunity to investigate new approaches to laboratory diagnosis, to evaluate and modify current laboratory processes to more accurately define a disease process, and to apply, by collaborative effort with interested physicians, the improved approaches to diagnosis and for evaluation of the physiological status of the patient at varying stages of illness.

Progress.

a. Application of microliter clinical chemistry procedures. In July 1967, the development of 26 clinical chemistry procedures for ultramicro determinations was completed. The amounts of sample required for valid ultramicro determinations range from 5 to 20 microliters. In July 1967, a study was established to determine normal values for infants and small children. Blood was obtained from heel punctures of newborn babies. Approximately 200 µl of blood was obtained from each baby on four consecutive days during the 12th, 36th, 60th, and 84th hours of life. The tests were performed in triplicate and at least two standards and one control were used with each determination.

The various analyses performed included: Total bilirubin, total protein, albumin, blood urea, nitrogen, true glucose (on whole blood, plasma, and serum). Total reducing substances (on whole blood, plasma, and serum). Presently, data from 48 babies have been obtained.

A complete evaluation of the data, including statistical analysis, is planned when complete data from 50 normal babies are available. It is anticipated that sufficient information will be in hand at that time for the publication of normal values for newborn infants.

b. A study of lipoproteins and serum lipids. The serum protein polysaccharide complexes were investigated as aids in the analysis of serum lipids, lipoproteins, and related materials.

It was previously shown that the amount of turbidity produced when dextran sulfate and calcium ions are added to serum is proportional to the total lipid level. It has been found that a serum standard, Moni-trol I, distributed by Dade Reagents, Inc., reacts similarly to serum by the same treatment. Since these Dade standards are accompanied by an assay sheet listing lipid components, a rapid method is available for the approximation of total lipids, cholesterol, and phospholipids. With each group of unknown sera, the Moni-trol standard is assayed by means of turbidity measurements of serial dilutions. The values for the unknowns are then estimated from a standard curve.

Since dextran sulfate precipitates most of the serum globulins, a study has been initiated to see if the precipitate contains thyroglobulin, the serum protein that transports thyroxine in the blood, which normally is present in blood in such a small amount it cannot be measured directly. Investigation is currently under process to evaluate the iodine content of the serum dextran precipitates and relate these to protein-bound iodine determinations in the diagnosis of thyroid functions.

c. Studies of spinal fluid. Investigations have been conducted regarding the biochemical changes observed in cerebrospinal fluid (CSF) in patients suffering from specific neurologic disorders. The studies were initiated in order to determine if certain correlations could be established between observed CSF changes and certain disease states. The establishment of such correlations would provide a useful diagnostic tool for the neurologist and hopefully permit some insight into the etiology of the demyelinating-type diseases.

The biochemical changes which were recorded included total protein, total lactic dehydrogenase (LDH) activity, total glutamic acid-oxalacetic acid transaminase activity, and electrophoretic analysis of CSF proteins and LDH isoenzymes.

A considerable number of problems regarding methodology were encountered and subsequently solved. These included concentration of the CSF specimen, stability of the LDH enzyme system, and quantification of protein electrophoretic patterns.

(1) Concentration of cerebrospinal fluid (CSF). The use of collodion tube vacuum dialysis (CTVD) has been demonstrated to be a quick and efficient method of concentrating dilute biological fluids such as CSF. CTVD was further developed and now represents a quick and efficient method of

concentrating dilute biological fluids such as CSF. The loss in total protein and gamma globulin observed with this system initially was eliminated by adding a process for conditioning fresh collodion tubes by reconcentrating three 5 ml aliquots of diluted (100-fold) serum standard prior to use.

(2) Quantitative analysis of protein electrophoresis patterns. Experiments were carried out to determine the validity of the quantitative analysis of protein patterns developed electrophoretically on glass slides containing 1% Nobel agar (agar gel electrophoresis), subsequently stained with 1% aniline blue-black dye in a 2% acetic acid solution.

Initially, excellent linear correlations were obtained with solutions of pure crystalline albumin between the amount of albumin on the slide and the densitometric response. The correlation appeared to exist within the range of 50 to 400 µg total albumin.

However, experiments with specifically prepared protein solutions containing known percentages of albumin and globulin, and possessing A/G ratios of 1.5, 2.4, and 4.0, demonstrated poor correlations between observed percentage values and known values at all three A/G ratios. Similar observations were made in repeated experiments with the agar gel-aniline blue-black dye system.

Another electrophoresis system was studied which utilizes cellulose acetate sheets as the stationary support and Ponceau S as the staining dye. The dye also contained small amounts of trichloroacetic and sulfosalicylic acids. Excellent results were obtained using this system to determine quantitative γ-globulin and albumin values, using standard protein solutions with concentrations ranging from 10 to 80 mg per 100 ml at A/G ratios of 1.5, 2.4, and 4.0.

All quantitative analyses of protein fractions were conducted with the cellulose acetate-ponceau dye system while the agar gel-aniline blue-black system was used for qualitative evaluation of the CSF proteins.

(3) Stability of lactic dehydrogenase (LDH) enzyme system. The stability of LDH activity in CSF specimens has been studied in some detail. There appears to be a rapid decrease in LDH activity of stored CSF at a temperature of -20°C, 4°C, and room temperature as measured by the rate of disappearance of NADH at 340 mµ or 366 mµ.

Contrary to previous findings in this laboratory, the ability of NADH to form a stable complex with the LDH enzyme system could not be demonstrated. This previously reported stabilizing effect of NADH was evaluated at temperatures ranging from 25°C to -20°C, using both the UV spectrophotometric method and colorimetric method. In all cases the activity was reduced to approximately 1/2 of initial activity within 24 hours.

Other compounds and substances were tested as stabilizers of the LDH enzyme system in CSF, but no suitable system could be developed. It was,

therefore, concluded that only fresh CSF specimens which could be obtained and analyzed within one hour following cervical or lumbar puncture would be utilized in present clinical studies.

Preliminary studies have also indicated a reduction in LDH following CTVD concentration of a CSF specimen for LDH isoenzyme analysis. However, it appears to be of little effect in loss of activity on the quantitative analysis of the isoenzymes.

Studies regarding the stability of CSF-LDH have been continued, however, with the hope that a more stable system may eventually be developed.

(4) Biochemical changes in cerebrospinal fluid (CSF) in patients with multiple sclerosis (MS). Although the number of CSF specimens which have been analyzed to date are insufficient for valid statistical analysis, specific biochemical changes resulting from Multiple Sclerosis and Encephalitis Periaxialis Diffusa (Schilder's Disease) are obvious. There appears to be no correlation of total protein and enzyme (GOT LDH) concentration between serum and CSF. It is also significant to note that LDH and GOT enzyme activity is consistently higher in serum than in plasma. It is believed that the higher values in serum reflect the contribution of platelets which rupture during blood clot formation. This observation serves to emphasize the significant alterations in CSF enzyme activities which may arise if all cells are not promptly removed from the supernatant via centrifugation.

A marked increase in total protein concentration in CSF from patients with "confirmed" multiple sclerosis was observed. The protein electrophoresis also indicates the dramatic increase in gamma globulin which characterized this disease. These changes were also observed in the single case of Schilder's Disease. The protein values of "probable" and "suspected" MS do not appear to vary significantly from those of normal subjects. This fact, together with the considerable variation in gamma globulin values obtained for the questionable patients, points up the need for more specific data to aid in diagnosing borderline cases.

The LDH activity of patients with "confirmed" MS and the single patient with Schilder's Disease was significantly lower than the normal group. The LDH activities obtained for the "probable" and "suspected" categories were more variable.

The isoenzyme patterns of "confirmed" cases and the "Diagnostic Problem" disclose a slight but significant elevation of LDH_3 as compared to the normal group which may also prove to be statistically significant with larger populations. The "Diagnostic Problem" subsequently was classified as a "confirmed" case of MS based on additional clinical data. Again, there was no clear indication of an elevation in the LDH_3 isoenzyme in the CSF of questionable cases of multiple sclerosis.

The investigation thus far points up the gross inadequacies of present biochemical methods used to aid with diagnosis and classification of the

demyelinating-type diseases. It appears that significant biochemical data are obtained only in cases where the disease has reached advanced stages and at a point when the diagnosis can be made on the basis of other clinical parameters.

Current investigations in this laboratory are concerned with the complete analysis of total CSF lipids with regard to lipid class and fatty acid pattern of each class. The methodology for extraction and quantitative thin-layer chromatography and vapor phase chromatography of the minute quantities of CSF lipids is presently being developed. Hopefully, characteristic changes will be observed which may be used independently or in conjunction with existing parameters in order to provide a useful tool for early diagnosis and differentiation of the degenerating diseases. It is also possible that such information may help to gain insight into the actual etiology of the demyelinating diseases.

Distinct changes in plasma levels of cholesterol, nonesterified fatty acids (NEFA), and triglycerides have been found during a four-week training period for experimental rats. Plasma cholesterol, at a high level in the untrained, exercised rat, decreased to approximately one-half the control levels in the rat trained for four weeks. In rats trained one, two, or four weeks, no significant changes in plasma cholesterol occurred after exercise. It thus appears that plasma cholesterol is lowered during this training schedule, but is not lowered significantly after a single exercise period.

When the untrained control rats were exercised, the NEFA were elevated. Exercise also resulted in elevated NEFA in the plasma of trained rats, except at two weeks of training when no significant increase after exercise was observed. These results indicate that the greatest changes in plasma NEFA after exercise occurred at the early and late stages of this training period.

The level of plasma triglycerides in the exercised rats was lower than that in the control. The triglycerides in the control rats were lower after one week of training, but remained relatively constant through the remainder of the training schedule. The triglyceride(s) in the exercised rats continued to decrease after one week to a minimum at the second week of training. These results suggest that the effects of exercise and training on plasma triglycerides are additive, the total effect depending on the amount of training the rat has received, with the maximum effect occurring during the middle part of this training schedule.

There was no significant change in the plasma levels of cholesterol esters and phospholipids from control levels with either exercise, training, or both.

A study of the biochemical variations in abnormal health states was continued with the Model E analytical ultracentrifuge. Investigation of proteins, lipoproteins, and abnormal globulins were continued, with emphasis on devising a method for mass handling of specimens and data.

The long-range study of lipoproteins as related to coronary disease, which was undertaken the latter part of January 1967, is continuing. At present 242 serum specimens have been received for lipoprotein analysis.

The lipoproteins are separated from serum and photographed with the Model E analytical ultracentrifuge, according to the method of Gofman. The resultant photographs are subjected to rigorous measurement and mathematical computation.

Because of the huge number of specimens, and the vast number of manipulations required in a study of this nature, several approaches to the project were tried. Initially, the photographs were enlarged, traced on a standard template, Xeroxed, cut out, weighed, and areas calculated. While this method gave good results, it was unsatisfactory from the standpoint of being extremely tedious and time-consuming in terms of the large volume of specimens.

In an attempt to expedite the task, additional rotors, cells, and a microcomparator have been added. The microcomparator has been adapted to the method of analysis. Although several months were required to achieve the transition to the microcomparator, because many additional factors had to be determined before complete adaptation was obtainable, the results obtained with the use of this instrument are most gratifying, the data handling substantially simplified, and the accuracy improved.

d. Protein technology and immunoelectrophoresis. A complete system of serum protein analysis has been put into operation and provides useful information for evaluation of the disease processes.

The system consists of:

(1) Colorimetric analysis of total protein and albumin and estimation of the A/G ratio.

(2) Agar gel electrophoretic analysis of serum proteins for qualitative evaluation of the patterns from various pathologic cases and quantitative determination of albumin and the globulins α_1, α_2, β_1, β_2, and γ.

(3) Immunoelectrophoresis of proteins for the qualitative evaluation of the immunoglobulins G, A, and M, as well as other proteins of special interest; e.g., lipoproteins α and β.

(4) Radial immunodiffusion for the quantitative determination of the immunoglobulins G, A, and M.

The combined application of these techniques provides detailed information on serum proteins in health and disease and a check against reagent and technician errors.

Information obtained to date has shown a correlation of serum protein patterns with clinical conditions which formed the basis for diagnosis and differentiation of pathological conditions.

Information on protein-lipid interactions in clinical conditions of an unusual nature such as the "Multiple Myeloma and the Nephrotic Syndrome," a biochemical and morphological study in collaboration with the Division of Medicine, WRAIR, has also been studied.

An unusual case of an 11-year-old female was referred for special protein studies. Serum and salivary proteins and immunoglobulins were determined. The data obtained did not fit any of the known cases of this laboratory or in the literature. A very recent publication has reported a newly discovered disease,"immunologic amnesia" reported for the first time, which appears identical to the case under study.

Data obtained by these techniques not only provide ready information as an aid in diagnosis, but also become part of the inventory of permanent records which serve as reference for interpreting other pathologic cases.

Determination of enzymatic activities in plasma is a useful method for studying metabolic changes of tissues in health and disease. Analysis of isoenzymes provides additional information because they may indicate the tissue origin of such changes. Lactate dehydrogenase (LDH) is present in all tissues and lends itself for studies of this nature. LDH is composed of five molecular forms called isoenzymes. A quantitative method for the determination of LDH-isoenzymes recently developed in this laboratory has been applied to studies of the effects of exercise and training in the rat.

First, a method for obtaining plasma samples from the rat was developed which eliminated the marked differences and variability of data reported in the literature. The degree of exercise was determined by swimming the rats at different time periods in order to achieve significant changes. After establishing the fact that stress by strenuous physical exercise produces significant enzymatic changes in the rat plasma, indicating metabolic changes in tissues and their origin, the degree and extent of training necessary to prevent these changes, or condition the rat against strenuous exercise, has been investigated.

It was found that training by swimming 4 hours daily for 4-6 weeks prevented the enzymatic changes in the rat plasma produced by strenuous exercise. Information obtained by these studies formed the basis of evaluating a military project "Study of Physiologic Alterations During Heat Stress and Exercise" carried out by the Department of Metabolism, WRAIR. Recent military experience has indicated an increase in the occurrence of heat stress injury in basic trainees prepared for duty in Vietnam. The Department of Metabolism undertook the clinical and chemical study of basic trainees. Serum samples were collected for LDH and isoenzyme determination before and after stress conditions, as well as in pathological cases of acute kidney failure. LDH changes could be correlated with the degree of training by the aid of the findings in the basic studies with the rat.

The basic technique of agar gel electrophoresis has been adapted for the determination of lipoproteins. Agarose proved a better medium than agar, and electrophoresis time was reduced to 10 minutes. This is a considerable

improvement over the currently used paper electrophoresis which requires about 16 hours. Analysis of normal human serum revealed 5 lipoprotein zones delineated on the microscope slide. The lipid composition of the lipoprotein zones is currently under investigation by chromatographic analysis.

e. Studies of vasoactive amines. The relationship of the vasoactive amines to the alterations of cardiovascular and pulmonary function in dogs that result from acute pulmonary embolism and cause death was investigated to determine what factors cause these alterations and to evaluate various means of medical therapy to prevent death due to massive pulmonary embolism. Dogs were anaesthetized and blood pressure, ECG, respiratory rate, tidal volume, and alveolar P-CO_2 were recorded. Blood samples for vasoactive amines and blood gases and cardiac output were collected. The animals were divided into controls and other sets pretreated with atropine, propranolol, or phenoxybenzamine, respectively, and given autologous clots until death, with blood samples being taken for amines, gases, and cardiac output at regular intervals before and after each clot.

This project has been completed. Insofar as the amines are concerned, i.e., histamine, serotonin, and the catecholamines, there was no evidence that they are implicated in pulmonary embolism.

A newly initiated study is designed to determine the effects of venoms from three families of snakes, Crotalidae, Elopidae, and Niperidae on the cardiovascular system, hemodynamics, and catecholamine levels in dogs and monkeys. Venoms are administered at twice the established LD_{50} I.V. with arterial blood pressure, heart rate, EKG, and respiration being continuously recorded. Samples for catecholamines and blood coagulation factors are taken at regular intervals. Work is in progress with some 150 catechol samples analyzed to date. Preliminary data indicate an early release of catecholamines into the blood stream by intravenous injection of venom.

f. Chromium in biological materials. Work has continued on the analysis of chromium levels in biological matter by atomic absorption spectrometry. The analytical method is the same as that described in previous annual reports. This long-range project is part of the varied programs of the Division on trace metals in nutrition and intermediary metabolism.

Human plasma and various dietary ingredients from different parts of the world were analyzed for their chromium levels. Likewise, all the dietary ingredients used for preparing "low" chromium rat diets were surveyed for their chromium levels. The chromium content of rat fetuses and organs was also determined in studies on placenta transfer of chromium and chromium distribution in mammalian tissue. Lastly, the chromium content of various extracts from yeast was determined routinely in studies of the nature of biological chromium and its interactions with insulin in carbohydrate metabolism.

g. Blood lithium determinations. Flame emission spectrometry was used to routinely analyze the lithium levels in human serum. The procedure was that described in the previous annual report. Lithium carbonate is used in large doses as a drug for the treatment of manic-depressive patients, and it is very important that rapid analytical methods be available for monitoring the blood lithium levels.

Summary and Conclusions.

A rapid method for the approximation of several serum lipids has been devised, using dextran sulfate along with a commercial serum standard. Plasma cholesterol and triglycerides decrease; non-esterified fatty acids increase; and cholesteryl esters and phospholipids remain unchanged in rats during a strenuous four-week training period.

Analyses by the ultracentrifuge can be expedited by the method utilizing the microcomparator (Gaertner) which appears to offer the best solution for the intricate manipulations required for the analysis of lipoproteins, macroglobulins, abnormal globulins, and the determination of floatation values, sedimentation coefficients, concentrations, molecular weights, and many other determinations associated with the measurement of physical properties in a biological system.

A diversified biochemical system has been developed of protein, isoenzyme, and lipoprotein analysis. It has been applied for basic and applied research in order to obtain information on physiologic and pathologic biochemical mechanisms and to provide information for the diagnosis and differentiation of disease. This system is being simplified for use in the clinical laboratory as well as in the field lab.

The vasoactive amines, histamine, serotonin, epinephrine, and norepinephrine do not appear to be involved in pulmonary embolism in dogs. They are released into the blood stream following intravenous injection of snake vemon.

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RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CB6427	CSGRD-1 65

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
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10a. CURRENT NUMBER/CODE: 61145011 3A0145018712 01 072

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) BIOCHEMICAL LABORATORY AUTOMATIC SYSTEMS

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
002300 BIOCHEMISTRY	11 67	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
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		CURRENT FY 69	4	100

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21. TECHNOLOGY UTILIZATION: LIFE SCIENCES

22. COORDINATION: NA

23. KEYWORDS: BIOCHEMISTRY, AUTOMATION, CLINICAL CHEMISTRY.

24.

(U) TECH OBJECTIVE - THE TECHNICAL OBJECTIVE OF THIS WORK UNIT IS TO PROVIDE A SYSTEM OF LABORATORY BIOCHEMICAL ANALYSIS THROUGH MODULIZATION OF AUTOMATIC WET CHEMISTRY ANALYSIS EQUIPMENT, DATA REDUCTION TECHNICLES, AND DEVELOPMENTAL EFFORT.

(U) APPROACH- AUTOMATED ANALYTICAL WET CHEMICAL MODULES ARE ASSEMBLED AND UTILIZED TO PROVIDE A MODEL SYSTEM TO ACCOMPLISH BIOCHEMICAL ANALYSIS.

25. (U) PROGRESS - JAN 68 THRU JUN 68 THE CLINICAL SECTION OF THE AUTOMATED LABORATORY FACILITY IS CURRENTLY CAPABLE OF PERFORMING NINETEEN DIFFERENT DETERMINATIONS. THE ESTIMATED MAXIMUM LEVEL OF PRODUCTION IS APPROXIMATELY 15,000 DETERMINATIONS PER WEEK WITH THE AID OF A CDS-70 INFOTRONICS SYSTEM AND SIX TECHNICIANS. THE DATA SYSTEM EMPLOYS A 12-CHANNEL ANTI-LOG TO DIGITAL CONVERTER WHICH IS CAPABLE OF AUTOMATIC CALIBRATION AND OF DETECTING, DIGITIZING AND PRINTING PEAK HEIGHTS IN TERMS OF UNITS PER UNIT VOLUME. SYNCHRONIZED OR NON-SYNCHRONIZED CHEMICAL ANALYZERS MAY BE USED. A 12-CHANNEL TYPEWRITER OUTPUT WITH A PAPER TAPE GENERATOR COMPOSES THE REPORT-PRODUCING FUNCTION. PROGRAMS ARE AVAILABLE TO CONVERT TAPE TO CARDS OR MAGNETIC TAPE FOR DATA PROCESSING AND STORAGE. QUALITY CONTROL AND STATISTICAL EVALUATION OF DATA ARE STRESSED. REPRODUCIBILITY OF DATA POINTS HAS BEEN EXCELLENT. THE SYSTEM WOULD APPEAR TO HAVE BROAD APPLICATIONS TO THE ROUTINE AND RESEARCH LABORATORY WITH A HEAVY WORK BURDEN. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
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DD FORM JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A014501B71P BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 01, Biochemistry

Work Unit 072, Biochemical laboratory automation system

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Description.

An automated clinical chemistry capability will be established with the immediate goal of providing an extended capability for the Department of Metabolism (Ward 30) activities and ultimate potential for a service function for the Walter Reed Army Institute of Research. This developmental activity will further evaluate data handling and procedures for incorporation into other Army Medical Department facilities.

Progress.

Sixteen analytical, chemical procedures are currently being performed utilizing Technicon AutoAnalyzers and a CRS-70 "Infotronics" digitizer. The digitizer automatically calibrates its response based on maximum peak heights of a series of standards produced by the AutoAnalyzer system. It then detects and prints out results, in terms of units per unit volume, from 12 simultaneously-operating synchronized or unsynchronized AutoAnalyzers. An example of the capacity of the system is shown in Table I. The results are transferred to a 12-channel typewriter output and also to a paper tape which is enterable into computer facilities for statistical evaluation and storage of data. In order to assure quality control of the procedures, standards are used to compare statistical parameters which include standard deviation, standard error and coefficient of variation. As shown by Table II, the system provides results with a high confidence factor for research evaluations.

TABLE I

DETERMINATIONS AVAILABLE ON CRS-70 SYSTEM

Test	per hr	Total/day	*Effective/day	Total/week	Effective/week
Na	40	240	168	1200	840
K	40	240	168	1200	840
CO$_2$	50	300	250	1500	1200
Cl	50	300	250	1500	1200
Creat.	40	240	180	1200	900
UN	40	240	180	1200	900
PAH	20	120	100	600	500
Inulin	20	120	100	600	500
Uric A.	40	240	180	1200	900
Phos.	40	240	180	1200	900
Glucose	40	240	168	1200	840
Ammonia	40	240	168	1200	840
Osmolarity		100	90	500	450
Total				14,300	10,810

*The term "Effective" indicates total samples processed less quality control samples.

TABLE II

PROCEDURAL EVALUATIONS

Test	Reference Value	Entries	Average	Variance	S.D.	S.E.	C.V.
Na	103± 5.0	62	103.2	2.0215	1.42	.18	4.1
	142± 3.0	30C	139.6	1.866	1.37	.25	2.9
K	33+ 2	61	33.4	0.1518	.39	.05	3.5
	4.3± .1	30C	4.15	0.0025	.05	.0091	3.6
Creat.	2.8± .3	167	2.75	0.0057	.075	.0058	8.2
	"	30C	2.76	0.0063	.079	.014	8.6
	1.0± .2	57	1.07	0.0030	.055	.0072	15.3
Bun	30.5± 2.0	62	30.6	0.3143	.56	.071	5.5
	"	30C	30.3	0.0628	.25	.046	2.5
Inulin	36.0	31	36.26	0.1544	.39	.071	3.2
	"	30C	35.60	0.0431	.21	.038	1.7
PAH	24.0	24	23.96	0.0502	.22	.46	2.8
		30C	23.77	0.0189	.14	.025	1.7
Uric A.	3.0	24	2.99	0.0007	.026	.0053	2.6
	5.1± .3	48	4.94	0.0147	.2	.017	7.4
PO_4	3.5± .2	22	3.47	0.0033	.057	.012	5.0

Average: Average as determined in this laboratory
S.D. Standard Deviation
S.E. Standard Error
C.V. Coefficient of Variation at ± 3 S.D. (99%)

The 30C entries represent a run of 30 consecutive samples of control serum. The entries without the C represent values obtained on the same or a similar control serum but taken from normal runs covering a period of approximately two months. In general, the latter controls were spaced every 10th or 15th sample in the routine run. The statistical values obtained from the 30C control series therefore indicate the variation during a specific run, while those from other entries indicate the variation from day to day.

The reference values for inulin and PAH were prepared in this laboratory and were aqueous solutions. A study is programmed to obtain statistical values on prepared urine and serum controls.

Statistical values for K, creatinine, and PAH were obtained at two widely different concentrations. While the coefficients of variation calculated at the two different concentrations are essentially the same for K and PAH, this is not true for creatinine. More studies are programmed to determine the variation of the coefficient of variation throughout the concentration range of each procedure.

Within the constraints imposed by lack of computer time to the laboratory for further program development, the system under investigation has demonstrated its value in handling large volumes of samples with good reproducibility of results.

Some difficulties remain within the CRS-70 system since it is a complex electronic device. Developments to date indicate full feasibility for incorporation of multiple channel clinical chemical analyses which may be assembled in a flexible arrangement with research quality results being consistently provided. Further work with additional procedures, instrumental assemblies, and analytical systems is planned for the near future.

Summary and Conclusions.

An automated chemistry capability which provides high precision clinical chemistry laboratory service for the Department of Metabolism, and offers an ultimate potential for a service function for the Walter Reed Army Institute of Research, has been established. The system, consisting of AutoAnalyzers, a typewriter, a paper punch, and an "Infotronics" digitizer, automatically calibrates its response and detects, digitizes, prints out and stores results of analyses with flexibility for choice of determinations being provided.

Publications.

None.

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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01 07 68	D. CHANGE 01 07 67	WHY U WRK U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER/CODE: 61145011 3A014501B71F 03 035

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) ECOLOGY AND CONTROL OF DISEASE VECTORS AND RESERVOIRS

12. SCIENTIFIC OR TECH. AREA: 002600 BIOLOGY
006900 ENVIRONMENTAL BIO 010100 MICROBIOLOGY

13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
09 54	NA	OTHER DA

16. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
PRIOR FY 68	3	60
CURRENT FY 69	3	60

17. PROCURE. METHOD: C. IN-HOUSE

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

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21. TECHNOLOGY UTILIZATION: PUBLIC HEALTH

22. COORDINATION: NA

23. KEYWORDS: ARBOVIRUS, DENGUE, CHIKUNGUNYA, TRYPANOSOMES, ECOLOGY, MOSQUITOES, VECTORS, TSETSE FLIES.

24.

(U) TECH OBJECTIVE - CONTROL OF ANTHROPOD VECTORS OF DISEASE OF MILITARY SIGNIFIANCE WITH EMPHASIS ON VECTORS OF ARBOVIRUS AND PARASITIC DISEASE. EFFORTS ARE ON INCRIMINATION OF VECTORS, HOST-PARASITE RELATIONSHIPS. LABORATORY AND FIELD STUDIES OF VECTOR POPULATION ECOLOGY. FINAL AIM IS THE UNDERSTANDING OF VECTOR BIOLOGY AND DISEASE TRANSMISSION IN ORDER TO APPLY CONTROL METHODS MOST EFFECTIVELY.

(U) APPROACH- MOSQUITO COLLECTIONS ARE MADE IN THE U. S. AND SOUTHEAST ASIA FOR ARBO-VIRUS ISOLATIONS AND STUDY OF MOSQUITO ECOLOGY. SYSTEMS ARE DEVELOPED FOR THE STUDY OF HOST-PATHOGEN-VECTOR RELATIONSHIPS INVOLVING MOSQUITOES - DENGUE AND CHIKUNGUNYA VIRUSES AND TSETSE FLIES AND TRYPANOSOMA BRUCEI AS MODELS. STUDIES ON INTERSPECIFIC COMPETITION INVOLVE AEDES AEGYPTI AND AEDES.

(U) PROGRESS - JUL 67 THRU MAY 68 A LABORATORY COLONY OF CULISETA MELANURA WAS ESTABLISHED BY THE FORCED INSEMINATION TECHNIQUE. EXPERIMENTAL TRANSMISSIONS OF HIGH MOUSE PASSAGED CHIKUNGUNYA VIRUSES WERE MADE. THE EFFECTS OF TEMPERATURE UPON VIRUS TRANSMISSION BY MOSQUITOES IS BEING STUDIED. AN EXPERIMENTAL MODEL FOR THE TRANSMISSION OF AFRICAN TRYPANOSOMIASIS BY TSETSE FLIES IS BEING DEVELOPED. ATTEMPTS ARE BEING MADE TO MAINTAIN INFECTIVITY OF TRYPANOSOMA BRUCEI BY INVITRO CULTURE FOR TSETSE TRANSMISSION. COMPARATIVE STUDIES OF THE BIOLOGY OF AEDES AEGYPTI AND AEDES ALBOPICTUS WERE MADE WITH EMPHASIS ON FACTORS CONTRIBUTING TO COMPETITIVE ADVANTAGE. FOR TECHNICAL REPORTS SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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Research 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 wide variety of studies of arthropods in the field and laboratory in relation to several groups of pathogenic organisms, as well as studies of the arthropods in their own right. The studies range from the ecology of arthropods to the transmission of disease organisms and the development of improved methods for study of arthropods.

Progress

1. Interspecific competition in mosquitoes

Additional observations were made on interspecific relationships in two species of Stegomyia in the field in Thailand, and in the laboratory. The field observations were conducted in conjunction with control studies discussed elsewhere in this report.

a. Field studies were conducted in cooperation with the Department of Entomology, U.S. Army Medical Component-SEATO on Koh Samui, an island in the Gulf of Thailand. The initial observations were of short duration, due to the necessity for initiation of planned control measures at the study site. The analysis of some of the observations was not complete at the time of this report, but preliminary results were promising enough to warrant a more extensive study. As noted below, the laboratory studies at WRAIR were conducted with colonies of widely separated geographical origins; therefore, it is quite possible that observations on the same life processes in the field with sympatric populations may yield quite different results. On Koh Samui the populations Aedes aegypti and A. albopictus have apparently existed side by side and interacted over a considerable period of time. It is assumed that aegypti was introduced into the area many centuries ago, no doubt at the same time as it was introduced to mainland SE Asia. Since introduction, aegypti has become abundant in urbanized areas where it is presumed to have "displaced" the already existing populations of albopictus. The biological mechanism for such displacement or replacement is unknown, as is the general sequence of events. Presumably, aegypti is better adapted to the water container habitats of domestic urbanized situations, but the nature of this adaptation is unknown. The larvae do not complete directly in the sense of predation, and in a small percentage of the habitats examined in the field the species may occur together and complete development together.

On Koh Samui, initial observations indicated almost complete separation of aegypti and albopictus breeding, with only some 8 per cent of the available containers holding larvae of both species. This is partially due to the albopictus failing to use containers inside houses, and the aegypti generally failing to go far from houses, but even in the zone of overlap of the species there were few containers inhabited jointly than would be expected by chance alone. An initial attempt was made to determine whether aegypti and albopictus larvae from the study village could be reared together in bamboo sections, a habitat usually occupied by albopictus, but not by aegypti. One hundred eggs were placed in each bamboo section in rain water and the section housed in a cage. Larvae were not provided with extra food, utilizing only what would normally be present in a rain filled bamboo section. The proportions of adults emerging from various proportions of the two species are given in table 1. The overall emergence rate was quite low for both species, probably due to the relative lack of nutritive material available, but the albopictus larvae seem better adapted to the bamboo habitat.

Since reproductive potential is an important part of the relationships among species in nature, observations were made on total egg production of aegypti and albopictus on Koh Samui, using females reared and offered blood meals after mating. The 33 female albopictus for which data are complete produced 57.2 eggs per mosquito, in one or two batches. Of the 1888 eggs produced 84.8 per cent hatched, a figure approaching that occurring in natural populations. The 20 aegypti females observed laid an average of 65.5 eggs per female, with a hatching rate of 91.4 per cent.

Laboratory studies on the displacement of albopictus by aegypti were continued, with primary emphasis on the comparative biologies of the two species. As in previous parts of the study, the Bangkok strain of aegypti and the Japan strain of albopictus were used.

b. Comparative biology of the egg stage

Eggs of the two species were collected, dried, and matured (conditioned) at 27 $\pm$ 2°C and 80% R.H. for varying periods of time. Samples of these eggs were then flooded with deoxygenated 10% hay infusion (in tap water, v/v) for 48 hours. The egg papers were then removed, dried, and held for one week, at which time the flooding procedure was repeated. Per cent hatches are shown in Table 2. Overall egg mortality (i.e., total of unfertilized eggs, eggs hatching prior to initial collection, and embryonated eggs which died or which failed to respond to the hatching stimulus) was consistently greater in albopictus. There was a much greater tendency for aegypti eggs to hatch in the first flooding, while albopictus eggs hatched rather evenly over the two floodings.

The median hatching times (HT_{50}) were determined for eggs of the two species using 14 and 17 day old eggs in deoxygenated 10% hay infusion. (Table 3) The HT_{50} for aegypti eggs is quite short, from one to four hours. A. albopictus eggs hatch much more slowly, requiring 26 to 57 or more hours for 50% hatch.

2. Comparative biology of the larval stage

Changes in growth rate and mortality in larvae of aegypti and albopictus were studied at two densities, five species ratios, and two temperatures (Tables 4 & 5). Median pupation time in hours (PT_{50}) and per cent mortality were determined for each sex of each species under the different regimes. Larvae of aegypti pupated earlier than albopictus larvae in all cases but one (1 of 20 comparisons). As the per cent of aegypti larvae was increased in the high density situation, both aegypti and albopictus larvae pupated later. This was not as pronounced in the low density experiments; only the albopictus larvae reared at 25° showed a consistent change in relation to the per cent of aegypti in the pan. These experiments show that both aegypti and albopictus larvae are inhibited in their growth by the presence of large numbers of aegypti larvae, at least at high population densities.

Mortality among larvae from the experiments described above is shown in Table 5. Overall mortality was quite low, even at high density, where larvae were subjected to poor nutrition as well as crowding.

3. Effect of larval environment on fecundity

Adult females from the larval biology experiments were offered a blood meal from a rabbit or a young chicken to test the effect of larval environment on fecundity (Table 6). There was no apparent relation between species ratio and numbers of eggs laid, nor was there any difference between females reared at the two temperatures ($p=0.36$). Females of both species laid fewer eggs when reared under crowded conditions, though aegypti females laid more eggs than albopictus females in most cases. There was a marked reluctance by albopictus females to feed on either the chicken or the rabbit.

4. Studies on growth inhibiting factors produced by A. aegypti

After observing the retarded growth of A. albopictus larvae when they were reared together with A. aegypti, experiments were designed to determine the nature of this inhibition. The active product was obtained from A. aegypti larvae placed under stress by rearing at densities of two or more larvae per sq. cm. (one or more larvae per ml.) under minimal conditions of nutrition (about 0.5 mgm food per larva, total). Water was collected at about ten days and the larvae removed and destroyed. Microorganisms were removed by passage through a Seitz filter, and the filtered water was stored at -18°C until used. Analysis for activity was made by bioassay against albopictus larvae. This consisted of eight to ten replicates for each treatment, with ten first instar larvae in 80 ml of the material being tested. PT_{50}'s were calculated for each treatment and analysed for significance by the monographic technique of Litchfield (1949. J. Pharmacol. Exp. Therap. 97:399-408.).

A portion of the data obtained to date are shown in Table 7. A basic difference is seen between water from pans in which larvae received

minimal food (Low food = 0.5 mgm per larva, approx.) and those in which larvae had ample food (High food = 5 to 10 mgm per larva). No inhibitory activity was noted in the latter case, even though the degree of crowding was the same in both cases. Another difference is seen in three separate experiments using untreated low food aegypti water. The difference in PT_{50} among the controls (about 24 hours) is probably due to temperature differences, while the change in relative retardation (Control Treatment = 0.7 to 0.9) is probably a reflection of the actual amount of active material in the particular sample being tested. Low food aegypti water was subject to heat distillation using a modified Vigreaux column and a variable reflux condenser (distance from liquid surface to product valve, 50 cm). The column was allowed to equilibrate for about 45 minutes before drawing off the distillate, which was removed at a rate of approximately one ml per minute. The distillate was arbitrarily divided into three fractions according to volume and pH. The first fraction (vapor-liquid equilibrium temperature 99.5°C) had a starting pH of 9.5, and end pH of 7.0, and contained approximately one third of the total volume. The second fraction (equilibrium temperature 100°), also about one third of the total volume, started at pH 7.0 and ended at a pH of about 5.0. The third fraction consisted of the undistilled residue. It had a pH of about 5, and contained large amounts of solids. These three fractions were bioassayed against albopictus larvae as described above, at both the full strength of the distillate ("x3") and at a dilution roughly equal to normal unfractionated aegypti water ("x1"). It is important to note that the dilutions are only approximations. Although the active substance apparently distilled over in the first fraction, the amount of material distilled decreases with decreasing concentration and a complete separation is not possible. It can be seen that this material is heat stable, distillable, and loses little, if any, of its activity following this treatment. The active fraction from heat distillation (Fr-1) has a strong amine-like odor, which led us to conclude originally that the active material was an amine. This seems unlikely, however, since the same odor is obtained from heat distillates of high food aegypti water and water with food but no larvae. It is also probable that the high pH of the first fraction is also due to this latter substance rather than to the active compound.

5. Effect on oviposition

We expected that aegypti water might repel oviposition by albopictus females, particularly if this material has any relation to competition between the two species in nature. In two separate tests of this hypothesis, aegypti water not only failed to repel albopictus females, but was more attractive than distilled water, albopictus breeding water, and water incubated with larval food only. It seems more likely at this time that the growth inhibiting compound functions in regulating density within populations of aegypti rather than in interspecific competition. However, this ability to inhibit growth of larvae of other species is likely to be of considerable importance in any situation where aegypti actively begins to colonize a new habitat already occupied by another species, as may be the case in recent

reports of aegypti larvae breeding in tree holes in the southern United States and the Caribbean.

6. Attempts at species hybridization

There have been reports of naturally occurring and laboratory hybridization involving aegypti and other Stegomyia species. Preliminary studies were undertaken to explore this aspect of the relationship of albopictus and aegypti. Reports of previous crossing experiments indicated that when these appeared to be successful the offspring resembled the female parent-raising males in the laboratory. The Stegomyia species are unusually adept in escaping from small openings in cages by crawling, and loose males in the insectary may even fertilize females through the mesh of the average cage. In the present series of experiments this possibility was excluded by double screening and other precautions. Some matings were made by the forced insemination technique to obtain as great a degree of control as possible. Forty matings of aegypti ♀ x albopictus ♂ were made by forced copulation, three by the reciprocal (albopictus ♀ x aegypti ♂). Eggs were produced in the first cross, but none of 677 hatched; no eggs were produced in the reciprocal cross. Eggs were also produced by females of both species allowed to mate naturally with males of the other species in cages, but none were fertile. Female mosquitoes offered blood meals frequently lay eggs without having mated, so this observation is not unexpected. However, all female aegypti exposed to natural mating with albopictus males did show sperm in the spermathecae on dissection.

c. Transmission of arboviruses by mosquitoes

Experimental transmission of dengue and chikungunya viruses was continued in a search for model systems for evaluation of the vector potential of Asian mosquito species to transmit these viruses. The original intent was to develop systems which would not require the use of primates. Transmission of both viruses by mosquito bite was effected last year, after infection of the mosquitoes by use of blood-virus suspensions in the membrane feeder. During the present year, work consisted chiefly of repetition of the transmission experiments under varying conditions of temperature of incubation, the confirmation of transmissions, and the use of a low mouse passage strain of chikungunya virus in further transmission studies with Aedes aegypti and A. albopictus.

Dengue virus transmissions involved both Stegomyia species and types 1 and 2 dengue virus, both of Bangkok origin. Mosquitoes were infected through the membrane feeder on rabbit blood to which had been added $10^{4\ 4}$ mouse LD_{50} virus per 0.02 ml., using type 1 virus. Two transmissions to suckling hamsters were obtained on day 21 after the infective blood meal, but transmissions on earlier and later days were negative. Mosquitoes became infected at reasonably consistent rates and retained the virus for up to 35 days, as measured by trituration and inoculation of aliquots of surviving mosquitoes; 24/36 aegypti and 16/26

albopictus yielding virus in this manner. The highest titer attained in aegypti was 3.9 logs, the highest in albopictus being 3.9. In general, the titers were much lower. The very low transmission rates detected in sucking hamsters (2/132 attempts with aegypti and 0/161 with albopictus) indicate that the system is not promising for the intended studies of transmission mechanisms. If the dengue transmission studies are continued, primates or tissue culture systems will be evaluated. Work with primates by others indicates that much higher transmission rates may be expected, offsetting the expense and difficulty inherent in the primate system.

Chikungunya transmission experiments were designed primarily to provide a system for testing the hypothesis that malaria and virus infections may interact in various ways in mosquitoes, including interference with the normal development of the malaria parasites. The early experiments were conducted with both Stegomyia species and virus of African origin which had undergone over 30 mouse passages. After infection on the membrane feeder, aegypti transmitted the virus to suckling mice 48 times, with mosquito incubation periods of up to 35 days. It appeared that environmental temperature at the time of infection of the mosquitoes might play a role in their ability to sustain and transmit the virus. However, a statistical analysis of the final data did not sustain this impression. Transmission appeared to be too erratic for use in the interference experiments and other systems will be evaluated for that purpose.

8. Studies on Glossina and transmission of trypanosomes

A colony of tsetse flies, Glossina austeni, has been established in the insectary for the laboratory transmission of Trypanosoma brucei. Due to the slow reproductive rate and technical problems in handling these insects, the colony is augmented through bimonthly shipments of tsetse pupae from Dr. T.A.M. Nash, Director, Tsetse Research Laboratory, Bristol, England.

The tsetse flies are maintained in groups of 10 in Geigy type cages and are offered blood meals daily from either rabbit ears or the flanks of guinea pigs. Pupae produced are incubated for 30 days at 28°C over moist sand. Emerging adults are added to the colony or used for transmission experiments. An average of 300 flies are reared weekly.

Earlier observations of Baker & Ward (in press) indicated that only tsetse flies 24 hours of age or younger could be infected with T. brucei group trypanosomes. This work is continuing and studies on the comparative infectivity of different vertebrate hosts to the flies have recently been started. To date, no infections have been observed in 78 flies fed on rats or mice exhibiting "Stumpy forms" of T. brucei (Lugala I). 6/87 flies fed on guinea pigs have had proventricular and midgut infections at day 30 after an infective feed. Previous work in London indicated that the infective trypanosomes reach the salivary glands of the tsetse flies about day 40. Older flies are being held for the period to confirm these observations.

Although polymorphic trypanosomes of the Trypanosoma brucei group may be cultured indefinitely on diphasic media such as Tobie's and Weinman's, infectivity to vertebrates and tsetse flies is usually lost within a day. The varying results of the infectivity of older cultures as described by Amrein have not been repeated by subsequent workers. The present studies have been initiated to find procedures for the in vitro culture of polymorphic trypanosomes utilizing tissue culture media and insect cell strains in place of the older blood-agar slants with various overlays. The Lugala I isolate of T. brucei, which is passaged through mice, rats, guinea pigs, and tsetse flies, is used for this investigation.

Most cultural procedures utilize dilutions of infected blood or centrifuged infected blood for obtaining trypanosomes for initiating cultures. It was observed that even with several washings of centrifuged parasites that not all the trypanosomes were concentrated and that some erythrocytes persisted. To provide a clear separation of trypanosomes, rabbit anti-rat or rabbit anti-mouse red and white cell serum was added to suspensions of infected blood to agglutinate the blood cells. With a 1:10 dilution of antiserum, agglutination was complete in 15 minutes. Brief centrifugation of the resuspended trypanosomes in M-199 gave pure parasite suspensions.

In an initial experiment, 1000 trypanosomes/ml were added to tissue culture flasks containing 6 ml of medium (NCTC #135 with 5% fetal bovine serum) and penicillin dihydrostreptomycin (100 units/100µg ml of culture) and incubated at 27°C. Control cultures were made in Tobie's medium. The cultures were transferred at 48, 72, and 96 hour intervals and two mice were inoculated IP with 1 ml of the culture at each transfer to check infectivity. All material from the Tobie's medium was negative at 48 hours. 48 hour cultures from the tissue culture media were infective but older cultures were non-infective.

A second experiment was similarly set up but used an initial suspension of 10,000 parasites/ml tissue culture fluid. These cultures were infective to mice at 72 hours but not at later periods. Trypanosomes were transferred at 48 hours into new flasks; half with NCTC #135 and 5% FBS and the other with the media supplemented by 0.5% Cercropia hemolymph. The former cultures were non-infective at 48 hours while those with added hemolymph were infective to mice at 72 hours.

Summary and Conclusions

1. Field and laboratory observations on the important dengue virus vectors, Aedes aegypti and A. albopictus, have indicated that their ecological relationships are extremely complicated. At this point there is no clear cut explanation for the seeming replacement of albopictus by aegypti in inhabited areas of SE Asia. Despite their great similarity in larval habitat and adult habits, there is a distinct cleavage between the species in the field, and it is difficult to see how direct competition operates in such circumstances. Laboratory experiments indicate that under some circumstances aegypti larvae may elaborate, presumably as part of their

metabolic processes, substances which retarded the growth of albopictus larvae. The effect may be noted even where the species are not present simultaneously. While retardation may not be as effective a tool as predation in competition, it still is a potent method in terms of population ecology. Additional field and laboratory experiments are planned with these and other species of mosquitoes.

2. Transmission of types 1 and 2 dengue virus was effected by Aedes aegypti and A. albopictus, using young hamsters as a vertebrate host. The rate of transmission and reproduceability of the system was, however, not sufficient to serve as a model for planned experiments in the mechanism of infections and possible effects of the mosquito hosts on the virus. Similarly, high and low mouse passage chikungunya strains were transmitted by aegypti in a search for a model system to explore the interrelationships of Plasmodium, mosquitoes, and viruses. In this case, also, the system developed thus far is not adequate for the projected study, which should ideally involve Anopheles mosquitoes and a mammalian Plasmodium species.

3. A colony of Glossina austeni has been maintained for transmission experiments with Trypanosoma brucei. These experiments are still in their early stages, but are aimed at a better understanding of the mechanisms of infection of the tsetse by the parasites, and the genetic relationships. As an adjunct to the study in tsetse, attempts are being made to maintain infective trypanosomes in tissue culture utilizing insect tissue culture medium. This will assist in maintaining the system in periods when tsetse may not be available.

TABLE 1

Percent of adult emergence from mixed populations of Aedes aegypti and A. albopictus reared in bamboo containers, Koh Samui, Thailand

Percentage of eggs in initial population			Adult emergence, percentage of possible population	
aegypti	albopictus		aegypti	albopictus
100	0		15.5	-
75	25		4.6	44.0
50	50		19.0	35.5
25	75		2.0	15.3
0	100		-	46.8
		Total	11.6	34.8

TABLE 2

Effect of conditioning period and number of floodings on egg hatching in A. aegypti and A. albopictus
200 eggs of each species for each age, divided into 10 replicates

		AEGYPTI			ALBOPICTUS		
No. days of conditioning	Flooding	No. of eggs hatched	% of final hatch	% of total eggs tested	No. of eggs hatched	% of final hatch	% of total eggs tested
1	1	48	52.2	24.0	14	15.4	7.0
	2	44	47.8	22.6	77	84.6	38.5
	Total	92	----	46.0	91	----	45.5
6	1	5	5.3	2.5	34	42.0	17.0
	2	89	94.7	44.5	47	58.0	23.5
	Total	94	----	47.0	81	----	40.5
15	1	186	99.5	93.0	87	63.4	43.5
	2	1	0.5	0.5	50	36.6	25.0
	Total	187	----	93.5	137	----	68.5

TABLE 2 (Continued)

Effect of conditioning period and number of floodings on egg hatching in A. aegypti and A. albopictus
200 eggs of each species for each age, divided into 10 replicates

No. days of conditioning	Flooding	AEGYPTI			ALBOPICTUS		
		No. of eggs hatched	% of final hatch	% of total eggs tested	No. of eggs hatched	% of final hatch	% of total eggs tested
20	1	180	99.5	90.0	73	62.4	36.5
	2	1	0.5	0.5	44	37.6	22.0
	Total	181	----	90.5	117	----	58.5
25	1	164	98.7	82.0	31	86.1	15.5
	2	2	1.3	1.0	5	13.9	2.5
	Total	166	----	83.0	36	----	18.0
29	1	189	99.0	94.5	71	67.0	35.5
	2	2	1.0	1.0	35	33.0	17.5
	Total	191	----	95.5	106	----	53.0

TABLE 3

Median hatch times (HT_{50}) for eggs of A. aegypti and A. albopictus in deoxygenated hay infusion, 27 ± 1° C

	Age of eggs (days)	HT_{50} (hours)	Confidence Limits (5%) Lower	Upper
aegypti	14	1.7	1.2	2.5
"	17	1.2	0.3	4.6
albopictus	14	32.0	26.4	38.7
"	17	41.5	30.1	57.3

TABLE 4

Median pupation times (PT_{50}) for females of Aedes aegypti and A. albopictus reared under different densities and species ratios

Experiment No. and temperature	% aegypti in pan	200 larvae/pan		1200 larvae/pan	
		aegypti	albopictus	aegypti	albopictus
I (25 ± 1° C)	100	133	---	260	---
	90	136	198	328	475
	50	137	180	215	450
	10	132	167	167	345
	0	---	159	---	278
II (24 ± 1° C)	100	202	---	510	---
	90	198	242	695	570
	50	196	208	520	560
	10	210	235	375	535
	0	---	232	---	530

TABLE 5

Per cent mortality in larvae and pupae of A. aegypti and A. albopictus reared under different conditions. Accuracy of starting numbers approx. ± 2.5%

Exp. No and Temp.	% aegypti in pan	200 larvae/pan		1200 larvae/pan	
		aegypti	albopictus	aegypti	albopictus
I (25 ± 1°)	100	1	--	6	--
	90	7	0	4	12
	50	13	3	5	11
	10	5	11	2	12
	0	--	7	--	10
II (24 ± 1°)	100	1	--	17	--
	90	9	0	28	10
	50	13	3	22	22
	10	5	11	0	15
	0	--	8	--	14

TABLE 6

Average numbers of eggs laid by female *aegypti* and *albopictus* raised under different larval regimes. (*None fed)

Experiment No. and temperature	% *aegypti* per pan	200 larvae/pan				1200 larvae/pan			
		Chicken		Rabbit		Chicken		Rabbit	
		AEG	ALB	AEG	ALB	AEG	ALB	AEG	ALB
I (25 ± 1°C)	100	118	---	127	---	62	---	75	---
	90	150	n.f.*	134	96	51	n.f.	51	n.f.
	50	154	n.f.	151	120	48	n.f.	56	n.f.
	10	118	115	152	102	72	43	36	74
	0	---	126	---	123	---	34	---	58
II (24 ± 1°C)	100	105	---	130	---	46	---	56	---
	90	157	n.f.	128	n.f.	49	73	60	94
	50	151	55	124	87	57	62	44	n.f.
	10	139	95	86	108	63	32	83	50
	0	---	110	---	106	---	35	---	49

TABLE 7

Changes in PT_{50} of albopictus females as a result of rearing larvae in aegypti water treated or obtained by several methods. Heat distilled and lyophilized samples compared with distilled water control, others with food water.

Source of water	Type of treatment	PT_{50} (hours) Control	PT_{50} (hours) Treatment	Significance ($p = 0.05$)
Lo food aegypti	None	220	318	*
" " "	"	197	212	*
" " "	"	196	214	*
" " "	1/2 strength	240	292	*
" " "	Heat dist. Fr-1, x3+	200	229	*
" " "	" " " x1	200	209	NS
" " "	" " Fr-2, x3	200	196	NS
" " "	" " " x1	200	194	NS
" " "	" " Fr-3, x3	200	204	NS
" " "	" " " x1	200	202	NS
Hi food aegypti	None	235	235	NS
" " "	Lyophilized, reconst.	175	179	NS
Food water control	None	238	220	*

+ Fraction 1, concentrated approximately threefold; etc.

PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04
Immunology

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA CX6432	CSGRD-1

4. PERIOD OF RESUME	5. KIND OF RESUME	6. SECURITY APT	6. SECURITY TID	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 68	B. CHANGE 01 07 68	U	U	NA	GA	A. WORK UNIT

10a. CURRENT NUMBER CODE
61145011 3A014501871F 04 015

10b. PRIOR NUMBER CODE

11. TITLE
(U) ANTIGEN-ANTIBODY REACTIONS IN VIVO AND IN VITRO

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
01C10C MICROBIOLOGY	08 63	NA	OTHER DA

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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 ISOLATION AND CHARACTERIZATION OF THE ENZYMES INVOLVED IN HYPERSENSITIVITY REACTIONS.

(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 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.

(U) PROGRESS - JUL 67 THRU JUN 68 THERE IS A REQUIREMENT FOR TWO SERINE ESTERASES IN THE CHEMOTAXIS OF RABBIT POLYMORPHONUCLEAR LEUKOCYTES. THERE IS A REQUIREMENT FOR THE ASSOCIATION OF TWO RABBIT GAMMA G ANTIBODY MOLECULES IN THE FIXATION OF COMPLEMENT BY IMMUNE COMPLEXES. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

TEXT NOT REPRODUCIBLE

26.

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Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

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

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Description.

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

Progress.

1. Chemotaxis of rabbit polymorphonuclear leukocytes

a. As summarized in the preceding Annual Report, evidence for a requirement for two serine esterases in the chemotaxis of polymorphonuclear leukocytes has been obtained using phosphonate ester inhibitors. One of them, termed the "activatable esterase," exists on the cell in an inactive form, and the chemotactic factor directly or indirectly transforms this esterase into an active enzyme. Evidence was also obtained suggesting that the activatable esterase was an enzyme with a particular affinity for aromatic amino acid derivatives. Based upon this suggestion attempts have been and are being made to directly demonstrate the activatable esterase using various aromatic amino acid esters as substrates.

b. The substrate tried was acetyl DL phenylalanine β napthyl ester. Histochemically, it was possible to show that the polymorphonuclear leukocyte split this substrate to a greater extent after the action of chemotactic factor than before. The difference was slight using rabbit peritoneal polymorphonuclear leukocytes and not always reproducible. It was much more definite employing rabbit polymorphonuclear leukocytes from rabbit peripheral blood, and even more clear cut using polymorphonuclear leukocytes from human blood.

c. Because of the limitations of histochemical techniques, attempts were made to repeat the above results using a biochemical assay. In this assay the β napthol liberated by the enzymatic splitting of the acetyl DL phenylalanine β napthyl ester is coupled to a diazonium salt and the concentration of the resulting dye read spectrophotometrically. Rabbit polymorphonuclear leukocytes were shown to have 3 esterases existing in an already activated state. One of them was completely inhibited by phosphonate esters at 10^{-8} to 10^{-9}M concentration on standing at room temperature for 15 minutes, the second was inhibited at 10^{-5} to 10^{-6}M concentration of the same phosphonates, and the third was not inhibited at 10^{-3}M by any of the phosphonates tested, even with longer times of inhibition.

d. The assay of esterase activity using acetyl DL phenylalanine β napthyl ester was difficult due to the ability of the cells to make β napthol unavailable to the diazonium salt, and the lack of relation of the degree of splitting to concentration of cells and time of incubation except at relatively low concentrations of cells. Only occasional evidence was obtained of a weak increase in activity following incubation of rabbit peritoneal polymorphonuclear leukocytes with chemotactic factor. Better reproducibility was found using human leukocytes but the increase in activity was neither great enough nor obtainable in a sufficiently controlled fashion to be able to demonstrate that the increased activity was in fact due to the activatable esterase. Attention is now being turned to other substrates with the hope of obtaining more definitive results.

2. Enzymatic mechanisms of phagocytosis

a. Phosphonate ester inhibitors of serine esterases were examined for their possible inhibitory effects on the phagocytic function of guinea pig polymorphonuclear cells. These enzyme inhibitors were found to interfere markedly with capacity of guinea pig cells to ingest sheep red blood cells sensitized with antibody and complement, in a manner compatible with the known specific enzyme - inactivating

properties of these compounds. The pattern of the relative inhibitory activity of various structurally related phosphonate esters on the phagocytic activity of guinea pig cells strongly resembled the pattern of inhibition for one of two esterases previously implicated by Ward and Becker in the complement-dependent chemotactic activity of rabbit polymorphonuclear cells. Experiments performed in conjunction with Dr. Peter Ward in which the effect of various phosphonate esters on complement-dependent chemotaxis and phagocytosis was examined within the same species (guinea pig) revealed an even closer similarity in the relative inhibitory activities of various phosphonates on these two different cellular functions. These experiments suggest that similar serine esterases may play a common role in several cellular functions. We are currently attempting to identify and characterize serine esterases involved in phagocytosis in rabbit polymorphonuclear cells in order to further compare enzymes involved in phagocytosis with those already characterized and involved in other cellular processes.

3. Lipid analysis of sensitized sheep erythrocytes lysed by guinea pig complement

Work on this problem has been temporarily discontinued due to lack of personnel.

4. The effect of chemical modification of the antibody on its complement fixing and other biologic activities

a. Taking advantage of the findings described in the preceding annual report, an amidinated and benzylated rabbit γG antibody was prepared and used to study the mechanism of complement fixation. The doubly conjugated antibody retained only 8 percent of the complement fixing ability of untreated antibody, but had not lost the ability to combine and precipitate with antigen. It partially suppressed the ability of untreated complement fixing rabbit γG antibody to fix complement, when the two were reacting together with antigen. This effect was explained by the requirement that two or more active (untreated) antibody molecules are required to be in close juxtaposition for complement fixation to occur. Under these circumstances, the presence of inactive (treated) molecules result in the failure of some of the active ones to associate, since there is random co-precipitation of the two types of antibody in the immune lattice. Based on this hypothesis a mathematical theory was devised which expressed quantitatively the degree of inhibition of complement fixation by varying the proportions of treated antibody to untreated antibody if 2, 3, or more molecules of antibody were required

to associate before complement fixation could occur. A quantitative experimental study of the suppressive effect in the light of this theory led to the conclusion that there is a requirement for two adjacent active γG-antibody molecules acting together in the fixation of complement.

5. The inhibition of complement dependent and non-complement dependent allergic reactions by derivatives of maleopimaric acid

a. The preceding annual report described the action of maleopimaric acid on the in vitro hemolytic complement activity of guinea pig serum. Because of its high potency in this regard it was desired to test maleopimaric acid as an in vivo inhibitor of complement dependent allergic reactions. Because of the toxicity of maleopimaric, recourse was had instead to fumaropimaric acid. Fumaropimaric acid is as potent a complement inhibitor as maleopimaric acid, but only one-third or less as toxic. The acute intravenous LD_{50} of fumaropimaric acid for guinea pigs is around 700 mg/kg.

b. When injected intravenously into guinea pigs in non lethal doses of 600 mg/kg, fumaropimaric acid blocks completely the cutaneous hemorrhagic Forssman reaction resulting from the intradermal injection of rabbit anti-sheep red cell antiserum, the antiserum being a source of anti-Forssman antibody. There is also complete inhibition by the same dose of fumaropimaric acid of the systemic, shock-like reaction resulting from the intravenous injection of the same antibody.

c. The reversed, passive Arthus reaction obtained following the injection of bovine serum albumin intravenously followed by the injection of rabbit anti-bovine serum albumin into the skin of guinea pigs was completely prevented by the prior intravenous administration of 600 mg/kg fumaropimaric acid. The inhibition of the reaction was complete at two hours, a time when the control untreated reaction was maximal, but had returned by three hours after the level of inhibitor had fallen.

d. Biopsies taken from the sites at two hours showed no infiltration with polymorphonuclear leukocytes. There was, however, deposition of antigen-antibody complexes and fixation of C'3 in the vessels of the animals given inhibitor which was the same as that found in the untreated controls.

e. In order to see if the inhibitory activity of fumaropimaric acid was restricted to complement dependent allergic reactions, a non-complement dependent allergic reaction was chosen for study. This reaction was the passive cutaneous anaphylactic (PCA) reaction produced in guinea pigs by both a rabbit antiserum against ovalbumin (heterocytotropic reaction), and fractionated, guinea pig γl antibody against ovalbumin (homocytotropic reaction).

f. Fumaropimaric acid given intravenously in 500 mg/kg concentrations gave marked (approximately 95%) but incomplete inhibition of the PCA reaction obtained with either antibody. The antihistamine pyribenzamine given in 100 mg/kg amounts gave the same degree of inhibition. Reduction of the dose of fumaropimaric acid below 500 mg/kg abolished its inhibitory effect. Fumaropimaric acid has antihistaminic activity, but this antihistaminic activity is present as low as 200 mg/kg, a concentration which has no visible effect on the PCA reaction.

g. In conclusion, fumaropimaric acid inhibits at least two complement dependent reactions, the Forssman reaction and the Arthus reaction. It also inhibits a non-complement dependent response, the PCA reaction. It is possible that the mode of inhibition of the complement dependent reactions is through complement but this requires further study to confirm. What the mechanism is of the inhibition of the PCA reaction by fumaropimaric acid is not known. Fumaropimaric acid activity is not restricted to inhibiting complement; it also has weak anticoagulant and antihistaminic actions.

6. Permeability globulins of human serum

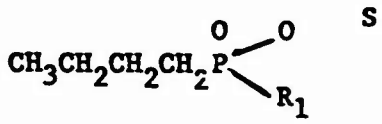
a. Pf/dil was purified 200 fold by acetone activation of human serum, followed sequentially by DEAE cellulose, CM-cellulose, and hydroxyl apatite chromatography. The partially purified enzyme was unstable and biologic activity was lost over a period of two weeks at 5°C.

b. The existence of two components of PF/dil in acetone activated human plasma was confirmed by electrophoresis on Pevikon block at pH 8.6 in 0.1M barbital buffer. Two distinct peaks of activity were isolated with one having approximately the mobility of albumin.

7. The synthesis of novel phosphonates

Twenty-one new phosphonates were synthesized. Their elementary analysis and physical properties are given in Tables I, II, and III.

TABLE I

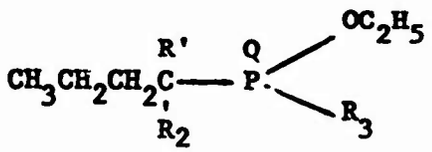


CYCLOPENTYL BUTYLPHOSPHONATES

Empirical Formula	R_1	B.P. °C		C		H		P		N/F	
				T	F	T	F	T	F	T	F
$C_{11}H_{23}O_3P$	Ethoxy	148/19	1.4448	56.4	55.0	9.9	10.5	13.2	13.0		
$C_9H_{18}ClO_2P$	Cl	82/0.01	1.4639	-	-	-	-	-	-		
$C_{15}H_{21}NO_5P$	p-$O_2NC_6H_4O$	144[A]	1.5206	55.2	55.0	6.5	7.2	9.5	9.3	4.3	4.3
$C_{14}H_{27}O_3P$	-OC_5H_9	121/0.15	1.4641	61.3	60.9	9.9	10.0	11.3	10.0		
$C_9H_{18}FO_2P$	F	62/0.05	1.4330	51.9	51.8	8.7	8.4	14.9	14.8	9.1	9.0

A. Falling film molecular still

TABLE II



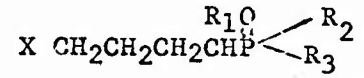
α - ALKYLPHOSPHONATES

Empirical Formula	R_1	R_2	R_3	BP°C	n_D^{25}	C T	C F	H T	H F	P T	P F	N/F T	N/F F
$C_{15}H_{22}NO_7P$	CH_3	CH_3COO	p-$O_2NC_6H_4O$	125[A]	1.5064	50.1	49.8	6.2	6.5	8.6	8.4	3.9	3.6
$C_{11}H_{23}O_5P$	"	"	C_2H_5O	66/0.1	1.4348								
$C_7H_{18}ClO_4P$	"	"	Cl	82/0.2	1.4478								
$C_9H_{18}FO_4P$	"	"	F	54/0.1	1.4226	45.0	45.1	7.6	7.5	12.9	12.6	7.9	7.6
$C_9H_{21}O_4P$	"	OH	C_2H_5O	44[B]									
$C_{10}H_{23}O_3P$	C_2H_5	H	"	69/0.1	1.4282	54.1	52.9	10.4	10.7	14.0	13.2		
$C_8H_{18}ClO_2P$	"	"	Cl	45/0.05	1.4452								
$C_{14}H_{22}NO_5P$	"	"	p-$O_2NC_6H_4O$		1.5215	53.3	53.4	7.0	7.2	9.8	10.0	4.4	4.4
$C_8H_{18}FO_2P$	"	"	F	87/20	1.4125	49.0	48.0	9.3	9.3	15.8	14.4	9.7	8.6

A. Falling film molecular still.
B. Melting point

TABLE III

MISCELLANEOUS BUTYLPHOSPHONATES



Empirical Formula	X	R_1	R_2	R_3	B. P. °C	n_D^{25}	C T	C F	H T	H F	P T	P F	F T	F F
$C_8H_{16}FO_4P$	H	CH_3COO	OC_2H_5	F	65/0.1	1.4137	42.5	46.2	7.1	8.4	13.7	13.0	8.4	7.7
$C_9H_{18}FO_4P$	"	$COOC_2H_5$	"	"	82/0.2	1.4187	44.7	43.9	7.5	7.6	12.9	12.7	7.9	7.8
$C_7H_{14}Cl_2O_3P$	"	"	Cl	Cl	61/0.05	1.4590								
$C_6H_{11}Cl_2O_3P$	"	CH_3COO	"	"	85/0.5	1.4600								
$C_{12}H_{22}ClO_4P$		"	"	OC_6H_{11}	120[A]	1.4662								
$C_{10}H_{24}NO_3P$	$(CH_3)_2N$	H	OC_2H_5	OC_2H_5	107/0.2	1.4388								
C_4H_8ClOP	O	H			78/0.2	1.4862								

A falling film molecular still

8. Chemical and biological characterization of homocytotropic antibody

a. Factors influencing the production of an anaphylactic antibody have been examined: dose of antigen, type of antigen, different adjuvants, route of administration, and the effects of splenectomy or treatment with 6 mercaptopurine. These variables were compared with the results obtained in 30 rabbits immunized by injection into the foot pads of 1.0 ml of a mixture of equal parts of DNP-BGG (20 mgm/ml) and complete Freund's adjuvant. Decreasing the immunizing dose to 1000 or 100 µgm results in a 50% fall in a number of animals producing homologous PCA antibody (approximately 1 in 4 as compared to 1 in 2). At an immunizing dose of 10 µg, the percent of animals positive remains about 25%, but the antibody can not be demonstrated until the 2nd or 3rd week, as compared with its usual appearance around day 7. At 1 µg only 1 of 8 animals was positive; the antibody appeared on the 13th day. The kind of antigen is an important determinant. Infection by schistosomes results in the production of high titers of anaphylactic antibody in 60 to 80% of rabbits, which persists for long periods of time. Egg albumin yields a slightly greater percentage of positives than does DNP-BGG, and somewhat higher titers of anaphylactic antibody. No differences were noted between 1 mgm and 5 mgm of antigen. There were no significant differences noted when incomplete Freund's adjuvant was substituted for complete adjuvant. Aluminum hydroxide gel appeared to increase the number and strength of positive reactions, but it was only studied with egg albumin as antigen. The effects noted could be ascribed to the antigen, rather than the adjuvant. The route of antigen administration was found to have an influence on homologous anaphylactic antibody production. The percent of rabbits which produced the antibody was similar following foot pad, intravenous or intraperitoneal injections of antigen, but rechallenge by the intraperitoneal route results in secondary, tertiary, and even quartinary antibody responses. This phenomenon of recall of anaphylactic antibody production has been seen with living antigens (e.g., schistosomes) but not with haptene or protein antigens given by the other routes.

b. Treatment of rabbits with 6 mercapto purine resulted in enhancement of 19S antibody, presumably by interfering with 7S antibody synthesis and its feed back inhibition of 19S antibody production. The effects observed on anaphylactic antibody production in rabbits receiving 10 mgm/kg of 6 MP included: decrease in % positive (3 of 14), but time of appearance and duration of demonstrable antibody was not significantly altered. These findings suggest that anaphylactic antibody production is

independent of conventional 19S and 7S antibody and is not under a feed back regulation by 7S antibody. At 20 mgm/kg, 7 of 8 animals were dead by the 13th day, up to that time no γG, γM, or anaphylactic antibody production was demonstrated. Splenectomies were performed in 10 rabbits. After a period of recuperation they were immunized with DNP-BGG (10 mgm) into the foot pads. γM antibody titers were higher than normal, and more prolonged. 7S antibody appeared later and in lower titers. Anaphylactic antibody was demonstrated in 6 of the 10 rabbits, the titers were not more elevated, but appeared to persist for longer than usual.

c. Attempts to isolate and characterize the rabbit anaphylactic antibody continue. Immunoelectrophoretic and Sephadex gel filtration studies have shown that the antibody is neither a conventional gamma G nor gamma M immunoglobulin. Dissociating it from the immunoglobulin A class has been difficult, because of the many characteristics which anaphylactic and γA antibody have in common. They migrate in a similar region on gel electrophoresis, appear to have similar molecular weights, and are eluted from DEAE cellulose columns with similar buffers. A specific sheep anti-rabbit γA antibody developed from rabbit colostrum does not remove anaphylactic activity. A guinea pig anti-rabbit anaphylactic antibody has been made by immunizing guinea pigs with a zinc sulfate fraction from a PCA positive serum. This antibody is not reac tive against rabbit γG, γM, or γA immunoglobulin. Absorption of rabbit serum containing anaphylactic activity by the guinea pig anti-anaphylactic antibody results in the complete removal of PCA activity from the rabbit serum. These studies, and others in progress, suggest that the rabbit anaphylactic antibody is not a γA globulin but may be representative of a heretofore undescribed class of rabbit immunoglobulins (γE immunoglobulin), analogous to the recent classification suggested for human reaginic antibody.

d. Methods for partial purification of the antibody have been developed. PCA activity is completely precipitated by saturated ammonium sulfate. When chromatographed with stepwise elution from DEAE cellulose with phosphate buffers (pH 8.0) of 0.01, 0.05, 0.10, 0.15, and 0.20 molarity, the PCA activity is eluted with the 0.1M fraction. Most recently it has been shown that the anaphylactic activity present in this fraction may not be homogeneous. When the ammonium sulfate precipitate from a serum containing anaphylactic antibody is chromatographed initially with 0.06M phosphate buffer (pH 8.0) and then eluted with a continuous gradient from 0.06 to 0.15M phosphate, the PCA activity is

found to reside in two separate areas. The first activity is in the fractions eluted at a conductivity of 8.5 - 9.0 mho's and the second at 12.5 - 13.0 mho's. The former region contains fast γG globulins, the latter the γA immunoglobulins. The activity of the first fraction does not appear to be removed by absorption with anti-anaphylactic antibody (anti γE?), the second fraction loses all of its PCA activity following this treatment. Studies on the heat lability of the two fractions, more complete characterization of the physical properties of each, and the factors which influence their elaboration are all areas of investigation projected for the coming year.

e. The characterization of mouse homocytotropic antibodies has been attempted with mouse antiserum obtained early after immunization and with sera taken from mice infected with T. spiralis. In both cases, at least two different antibodies seem to be present: one able to induce PCA only when a short latent period is used (4 hours), and another able to remain in the skin for more than 72 hours. In addition, as will be described, there is some evidence for a third antibody.

f. In mice infected with T. spiralis both antibodies appeared in the circulation five weeks after infection and reached their highest level around the ninth week. A secondary infection resulted in an increase in the level of both antibodies. In animals submitted to repeated reinfections the reagin-like antibody disappeared from serum while antibody responsible for the 4 hour PCA increased.

g. Immunization of mice with an extract of T. spiralis larvae or with ovalbumin induced the production of the reagin-like homocytotropic antibody, although at a low level. Separation of the 4 hour PCA Ab from the 72 hour PCA Ab has been performed on a DEAE-cellulose column using a gradient elution system. The antibody activity recovered in the first protein peaks was able to induce PCA after 4 hours but not after 72 hours; later eluates contained the activity responsible for the 72 hour PCA. Treatment with mercaptoethanol or heating at 56°C caused partial inactivation of the activity present in the first eluates and total inactivation of the activity present in the last eluates. Furthermore, some of these eluates were not able to induce PCA in rats although able to induce PCA in guinea pigs. Taken together, these experiments indicate the presence of at least three homocytotropic antibodies in mice. Presently,

mouse antisera containing reaginic antibody are being submitted to zone electrophoresis. Preliminary results indicate that mouse reaginic antibody is a fast migrating immunoglobulin.

10. Histamine release from rabbit platelets

a. Well washed pure platelet suspensions from rabbits infected with Schistosoma mansoni were unable to release histamine on the addition of specific antigen extract of S. mansoni. The presence of leukocytes from infected rabbits were needed in order to obtain antigen-induced histamine release. Leukocytes from non-infected rabbits mixed with platelets from infected rabbits were inactive, although platelets from normal rabbits used with leukocytes from infected rabbits were able to sustain histamine release on the addition of antigen. This indicates that only the leukocytes are sensitized. It was also noted that antigen-induced histamine release was possible from leukocytes from infected rabbits in the absence of platelets.

b. Experiments were conducted to discover the cell type in the leukocyte fraction responsible for this interaction with platelets. The leukocyte fraction was always contaminated with variable proportions of red cells. When red cells were obtained from infected rabbits which were free of leukocytes and tested with normal platelets no histamine release was obtained on the addition of antigen.

c. Neutrophils obtained from peritoneal exudates from infected rabbits were unable to sustain antigen-induced histamine release with normal platelets. Macrophages obtained from the peritoneal exudates of infected rabbits were also unable to sustain antigen induced release with normal platelets.

d. Different types of leukocytes from infected animals were separated by their adhesive capacity on siliconized glass beads and tested for their ability to interact with platelets from normal rabbits. The results obtained clearly established that pure suspensions of lymphocytes were capable of eliciting antigen-induced histamine from platelets. Further, the amount of histamine release was found to be linearly related to the amount of pure lymphocytes added to a constant amount of platelets.

e. The isolation of pure neutrophils from peripheral blood on glass beads has not been successful. Invariably the neutrophil fractions are contaminated with lymphocytes. These preparations of neutrophil

fractions cause histamine release which can not be wholly accounted for by its contamination with lymphocytes. These experiments indicate the following possibilities; 1) Although pure lymphocytes can interact with platelets to give antigen-induced histamine release the presence of neutrophils enhances this interaction; or 2) neutrophils are capable of the same interaction with platelets as lymphocytes though not necessarily through the same mechanism; and 3) the lymphocytes contaminating the neutrophil fractions are much more active than those isolated with the lymphocyte fractions. Further work is planned to distinguish among these possibilities.

11. Studies on blood group antigens and antibodies

a. The Wurmsers have concluded on the basis of extensive work that the specific binding properties of the anti-B isohemagglutinins from the sera of normal individuals are characteristic of the genotype of the ABO blood group of the donor of the serum. They also concluded that all individuals with a given ABO genotype have anti-B isohemagglutinins which are homogeneous in their specific binding characteristics (Prog. Biophys. 7: 88, 1957). Because of the theoretical importance of these claims and their potential practical importance in forensic medicine, the observations of the Wurmsers have been investigated.

b. In this investigation, the earlier observations made with a single group A_1 and group A_2 serum were confirmed with two other low titered group A_1 and three group O sera. In these studies, the binding properties of human, naturally occurring anti-B isohemagglutinins were determined by reacting to equilibrium a constant amount of a given serum with 5×10^5 to $1.5 \times 10^6/mm^3$ concentration of B cells, the same high concentration of B cells used by the Wurmsers. The antibody free in the supernatant and that fixed to the B red cell antigen were measured using the log probit assay of Wilkie and Becker. For all sera, the ratio of the amount of antibody fixed per cell to the amount of antibody free in the supernatant after adsorption was plotted against the amount of antibody fixed per cell. In all cases, a straight line was obtained parallel to the abscissa, that is of zero slope. In addition, the same data were used to plot the reciprocal of the antigen fixed per cell against the antibody free in the supernatant; this is the same method of treating the data as the Wurmsers employed. All sera gave straight lines of positive slope with this method. The latter finding was in agreement with the claims of the Wurmsers, that under their conditions of study a homogeneous antibody population was being tested. However, when cell concentrations greater than $1.5 \times 10^6/mm^3$ were used to absorb the sera, binding curves plotted

according to the Wurmsers' method deviated from linearity. The limited range of the linear portion of these binding curves indicates that the natural anti-B population of apparent homogeneity with which the Wurmsers are dealing is a fraction of unknown size of the total heterogeneous antibody population.

c. In the earlier investigations, the Wurmsers' findings that there are characteristic differences in the binding properties of anti-B isohemagglutinins from individuals of different ABO genotypes could not be confirmed using relatively low concentrations of B cells (3,000-10,000/mm^3) to absorb the sera or when undiluted serum was absorbed with relatively high concentrations of cells (500,000-1,500,000/mm^3). The binding properties of the isohemagglutinins of five group O, two group A_1O, and four group A_2 sera have been re-investigated using 500,000 to 800,000 cells/mm^3 to absorb constant amounts of a dilution of the antiserum at 37°C and 25°C. Moreover, the Wurmser assay technic was used along with the log-probit assay method except that there were quantitative differences in the slopes and positions of the lines. However, when a proportionality constant was used to convert the log-probit data to the same basis as the Wurmser data, the converted log-probit data fitted the Wurmser assay lines. These findings provide the first independent evidence in support of the conclusions of the Wurmsers' and also show that the log-probit assay method can be used to obtain the same results as are obtained with the more tedious and time-consuming Wurmser assay technic.

2. Evaluation of the Coulter Model B electronic cell counter for enumeration of free cells in agglutination at 4°C

a. The technic of counting free cells in agglutinated samples, reported by Gibbs et al for hemagglutination systems at 25°C, was modified and applied to B-anti-B hemagglutination equilibrated at 4°C. The preliminary evaluation of the free-cell data obtained by the instrument at 4°C showed excellent correlation with the data obtained by the visual hemacytometer cell counting technic. However, the method may not be practical since the fused aperture tube cracked after repeated exposure to the cold saline suspensions of red cells. Cemented aperture tubes are presently being tested for durability under the conditions of the test.

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

a. The following commercial vaccines were tested for the presence of blood group A and B activity: 35 Plague, 6 Influenza Virus, 6 Cholera,

2 Epidemic Typhus, 2 Rabies, 1 Monovalent Typhoid, and 2 Diptheria-tetanus toxoids. These vaccines had no detectable A or B blood group substances.

b. Freeze-dried crude preparation of blood group substances was prepared from the gastric mucosa of 40 hog stomachs showing blood group A substance activity and from 19 horse stomach linings showing blood group B substance activity. These materials were sent to Dr. George Springer, Northwestern University for purification of the respective blood group substances. The purified products are to be used as the standard preparations for testing the blood group A and B activities of commercial vaccines.

Summary and Conclusions:

1. There is indirect evidence that the "activatable esterase" of chemotaxis is an enzyme with a particular affinity for aromatic amino acid derivatives.

2. There is a histochemical evidence that following the action of the complement dependent chemotactic factor C'(5,6,7)a there is an increase in the ability of the polymorphonuclear leukocyte to break down acetyl DL phenylalanine β napthyl ester.

3. Two adjacent γG rabbit antibody molecules are required to fix guinea pig complement.

4. Fumaropimaric acid inhibits both the cutaneous hemorrhagic reaction induced by injecting rabbit anti-Forssman antibody into the skin of guinea pigs and the systemic reaction caused by the intravenous injection of the same antibody. The reversed passive Arthus reaction in guinea pig skin is also prevented by fumaropimaric acid. The same inhibitor partially prevents the PCA reaction induced by rabbit antiserum or guinea pig γ1 antibodies.

5. The existence of two components of PF/dil in acetone activated human plasma was confirmed by Pevikon block electrophoresis.

6. Twenty one new phosphonates were synthesized.

7. Treatment of rabbits with 6 mercaptopurine resulted in a decrease of the percentage of animals giving homocytotropic antibody.

8. Evidence for three mouse homocytotropic antibodies was obtained.

9. Leukocytes obtained from rabbits infected with Schistosoma mansoni were found to both specifically release histamine and to be required for the release of histamine from platelets of infected or uninfected rabbits on the addition of antigen. Lymphocytes from infected rabbits gave no antigen-induced histamine release themselves, but were capable of interacting with platelets to induce the latter to do so when antigen was present.

10. There is a natural anti-B blood group population of homogeneous binding affinity in human sera which is a fraction of unknown size of the total heterogeneous anti-B isohemagglutinin population.

11. The conclusions of the Wurmsers that these are characteristic differences in the binding properties of anti-B isohemagglutinins from individuals of different ABO genotypes were confirmed.

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2. Becker, E.L. and Ward, P.A. Partial biochemical characterization of the activated required in the complement dependent chemotaxis of rabbit polymorphonuclear leukocytes. J. Exp. Med. 125: 1021, 1967.

3. Becker, E.L. The relationship of the structure of phosphonate esters to their ability to inhibit chymotrypsin, trypsin,acetlyl cholinesterase and C'1a. Biochimica Biophysica Acta 147: 289, 1967.

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10. Smith, J.K. and Becker, E.L. Serum complement and the enzymatic degradation of erythrocyte phospholipid. J. Immunol. 100: 459, 1968.

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15. Pearlman, D.S. and Becker, E.L. Inhibition of phagocytosis by phosphonate esters. Fed. Proceed. 27: 249, 1968.

16. Glovsky, M.M., Ward, P.A. and Becker, E.L. Inhibition of complement-dependent biologic reactions by fumaropimaric acid. Fed. Proceed. 27: 313, 1968.

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18. Gibbs, M.B. Studies of the thermodynamic properties of naturally occurring anti-B isohemagglutinins. Fed. Proceed. 27: 1189, 1968.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	4. REPORT CONTROL SYMBOL
		DA OA6433	CSCRD-L03

5. DATE OF RESUME	6. KIND OF RESUME	7. SECURITY	8. REGRADING	9. RELEASE LIMITATION	10. LEVEL OF RESUME
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11a. CURRENT NUMBER/CODE: 61145011 3A0145018719 04 016

11b. PRIOR NUMBER/CODE:

11. TITLE: (U) IMMUNIZATION STUDIES OF EXOTIC DISEASES

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

16. PROCURE. METHOD	17. CONTRACT/GRANT			18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
	a. NUMBER	b. TYPE	c. AMOUNT			
C. IN-HOUSE	NA	NA	NA	PRIOR FY 68	2	80
				CURRENT FY 69	2	80

19. GOVT. LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION: PUBLIC HEALTH
CIVIL DEFENSE

22. COORDINATION: NA

23. KEYWORDS: CHIKUNGUNYA, VACCINES, IMMUNOLOGY, TISSUE CULTURE.

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.

(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.

(U) PROGRESS - JUL 67 THRU JUN 68 VACCINE AND STOCKS SUITABLE FOR USE IN THE PRODUCTION OF HUMAN VACCINES HAVE BEEN PREPARED IN CERTIFIED BANK-FROZEN GREEN MONKEY KIDNEY TISSUE CULTURE, UTILIZING HUMAN ISOLATES OF THE CHIKUNGUNYA VIRUS. THIS PROCEDURE HAS ELIMINATED THE POTENTIAL HAZARD OF ADVENTITICUS MURINE AND SIMIAN AGENTS IN THE FINAL VACCINE PRODUCT. CURRENT RESEARCH IS ALSO IN PROGRESS TO DETERMINE THE SUITABILITY OF HUMAN DIPLOID WI-38 CELLS FOR USE IN THE PRODUCTION OF VACCINES FOR HUMAN USE. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

25.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. CONFID OR CONFID 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.

DD FORM 1 JAN 66 1498m REPLACES EDITION OF 1 JUN 65 WHICH MAY BE USED (Items 1 to 26 identical to NASA Form 1122)

Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 04, Immunology

Work Unit 016, Immunization studies of exotic diseases

Investigators.
Principal: V. R. Harrison, MS
Associate: K. Eckels, BS; C. Hampton, BA

Description.

This task is concerned with the development, production, and evaluation of either live-attenuated or formalin-killed vaccines against exotic viral agents.

Progress.

Vaccines and virus seed stocks for the arthropod-borne group are usually prepared from material derived from young-adult or suckling mouse brain. Since most tissues of murine origin are known to harbor a host of adventitious agents, we have attempted to by-pass this hazard by the direct isolation and passage of the chikungunya virus from infected human serum in a non-murine tissue culture system certified-free of detectable adventitious agents.

For this purpose we have utilized the bank-frozen kidney cell suspension of one cercopithecus monkey. Twenty-five percent of the total volume of the trypsinized harvest from this monkey was exhaustively tested for the presence of simian adventitious agents, according to methods prescribed by Public Health Service Regulations for Inactivated Poliomyelitis Vaccine (Title 42, Part 73, Rev. 1965), with negative results. The remaining 75% of the suspension was stored frozen under liquid nitrogen with 5% glycerin as the cryoprotective agent. For use in virus passage, ampoules of these frozen cells were thawed and monolayers prepared as requested.

Four human sera infected with the chikungunya virus and collected by Dr. S. B. Halstead during the 1962 outbreak in Thailand were used as the source virus. Direct isolation and identity tests were made in the monkey kidney tissue culture described above and each isolate was then passaged 10 times at a dilution of 1:100 in these cells. Vaccines were prepared with each isolate at the 5th and 10th passage level, respectively, and assayed for potency.

TABLE 1

Data on vaccines prepared with human CHIK isolates at the 5th and 10th tissue culture passage levels

Isolate and Vaccine No.	Passage Level	Harvest		Potency*
		LD_{50}/0.02 ml	HA Titer 1:-	ED_{50}/ml
6348	5	4.8	128	0.42
6461	5	5.8	128	0.46
15561	5	5.4	128	0.18
23337	5	5.9	128	0.37
6348	10	6.0	32	0.31
6461	10	6.3	32	0.53
15561	10	6.5	32	0.20
23337	10	5.9	32	0.30
Ref. Vaccine Lot E-12	---	6.2	64	0.13

* Challenge consisted of the intracerebral inoculation of ≈ 250 LD_{50}s.

From the data presented in Table 1 it may be seen that these vaccines conferred significant levels of protection to mice against an intracerebral challenge with the high passage African strain 168 CHIK virus.

To determine the efficacy of CHIK vaccine prepared in bank-frozen monkey kidney tissue culture against a serologically-related member of the CHIK complex, a vaccine trial was performed in rhesus monkeys, using the CHIK 168 and Mayaro virus in the challenge procedure. Six male rhesus monkeys were given a 1.0 ml subcutaneous injection of vaccine on days 0, 7 and 21. Six additional monkeys were held as non-vaccinated controls. Thirty days after receiving a primary series of vaccine the monkeys were divided into 2 groups comprising 3 vaccinated and 3 control animals each. One group was injected with ≈ 7500 LD_{50}s of the CHIK 168 virus while the other received ≈ 30,000 LD_{50}s of the Mayaro virus. Prior to challenge the vaccinated monkeys were bled to determine their serologic response to vaccine. These data are shown in Table 2.

TABLE 2

Monkey No.	Antibody levels 30 days after vaccination		
	Neutralizing Index	Complement-Fixation	HI
679	2.86	4	40
697	2.07	<4	40
698	2.22	<4	20
699	2.37	<4	40
721	2.37	8	40
905	2.43	8	80

All monkeys were bled on 6 consecutive days after challenge to determine plasma viremia levels. From the data in Table 3 it may be seen that none of the vaccinated monkeys challenged with the CHIK 168 virus developed viremias, while 2 out of 3 vaccinated monkeys were protected against a viremia when challenged with the Mayaro virus, indicating that the vaccine does afford some protection against a heterologous virus within the CHIK complex.

TABLE 3

Monkey No.	Vaccine Status	Challenge Virus	Viremia on day post inoculation				
			1	2	3	4	5
679	Yes	CHIK	0	0	0	0	0
697	Yes	CHIK	0	0	0	0	0
905	Yes	CHIK	0	0	0	0	0
698	Yes	Mayaro	2.0*	2.0	1.5	0	0
699	Yes	Mayaro	0	0	0	0	0
721	Yes	Mayaro	0	0	0	0	0
651	No	CHIK	0	0	0	0	0
671	No	CHIK	0	1.0	1.7	0	0
702	No	CHIK	1.9	2.7	1.0	0	0
703	No	Mayaro	2.0	2.0	2.7	0	0
708	No	Mayaro	3.5	4.0	3.4	0	0
742	No	Mayaro	2.6	3.6	3.3	0	0

* $TCID_{50}$/0.1 ml

From the data presented it may be seen that CHIK vaccine prepared from bank-frozen monkey kidney tissue culture elicits significant levels of protection in mice and rhesus monkeys.

The recent highly fatal infection reported in technicians handling cercopithecus monkey kidney tissues (Marburg and Frankfurt, Germany, 9 Sep 67) suggests that there are yet hitherto undefined simian adventitious agents. This most recent outbreak lends strong support to the contention that the number of simian adventitious agents is limited only by the degree of refinement in detection techniques. Thus, as the problems associated with the manufacture of vaccines in tissue culture for human use increase, there is a corresponding decrease in the variety of tissue culture systems available for this purpose.

The generally increasing acceptance of the highly characterized human embryonic cell line, designated Wi-38, has indicated that this may be the most suitable substrate for the manufacture of vaccines for human use in the near future. It is on this premise that we are currently studying the feasibility of using Wi-38 cells for the production of CHIK vaccine. Adaptation of the previously described CHIK isolates to the Wi-38 human diploid cell line is in progress.

Publications.

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3. V. R. Harrison, L. N. Binn and R. Randall. Comparative Immunogenicities of Chikungunya Vaccines Prepared in Avian and Mammalian Tissues. Am. J. Trop. Med. & Hyg., Vol. 16, 1967.

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 CA6437	CSCRD-103

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

10. CURRENT NUMBER/CODE: 61145011 3A014501B71P 08 075

10b. PRIOR NUMBER/CODE:

11. TITLE: (U) CELL GROWTH AND REGENERATION

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
012900 PHYSIOLOGY 016200 STRESS PHYSIOLOGY 002300 BIOCHEMISTRY	09 58	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In Thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	PRIOR FY 68	3	60
		CURRENT FY 69	3	60

19. GOVT. LAB/INSTALLATION/ACTIVITY

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21. TECHNOLOGY UTILIZATION: CLINICAL MEDICINE
TRAUMATIC SURGERY

22. COORDINATION: NA

KEYWORDS: WOUND HEALING, CELL CULTURE, FIBROBLAST PROLIFERATION, COLLAGEN BIOSYNTHESIS, ENERGY METABOLISM, ATP, GLUCOSE AVAILABILITY, OXYGEN TENSION.

24.

(U) TECH OBJECTIVE - THE DEFINITION OF THE CELLULAR MECHANISMS CONTROLLING WOUND HEALING AND TISSUE REPAIR FOLLOWING COMBAT INJURY SUCH AS MECHANICAL, THERMAL, OR CHEMICAL TRAUMA, INFECTION, IONIZING RADIATION, AND SHOCK.

(U) APPROACH- TO MAXIMIZE EXPERIMENTAL CONTROL AND ANALYTICAL RESOLUTION, A WELL DEFINED IN VITRO SYSTEM IS USED TO STUDY FIBROBLASTIC CELL POPULATIONS UNDER NORMAL AND SIMULATED TRAUMA CONDITIONS THROUGH THE APPLICATION OF A WIDE SPECTRUM OF BIOLOGICAL PHYSICAL CHEMICAL PROCEDURES.

(U) PROGRESS - JUL 67 THRU JUN 68 IT WAS FOUND THAT UNDER THE PROPER CONDITIONS FIBROBLASTS GROWING IN VITRO PROCEED FROM A PHASE CHARACTERIZED BY A HIGH RATE OF CELL DIVISION AND MINIMAL COLLAGEN SYNTHESIS TO A PHASE CHARACTERIZED BY MINIMAL CELL DIVISION AND A HIGH RATE OF COLLAGEN SYNTHESIS. THE EVOLUTION OF THE FIBROBLASTIC CELLS IN THIS IN VITRO SYSTEM WAS THUS SHOWN TO PARALLEL THE MATURATION OF A WOUND IN THE BODY. IN A FURTHER SEARCH FOR THE DETERMINANTS OF THIS EVOLUTION IT WAS DEMONSTRATED THAT THE ARREST OF CELL DIVISION AND THE INITIATION OF COLLAGEN BIOSYNTHESIS WAS PRECEDED BY A MARKED DECREASE OF THE LEVEL OF THE HIGH ENERGY COMPOUND ADENOSINE TRIPHOSPHATE (ATP) IN THE MATURING FIBROBLASTS. THE MOST OBVIOUS FACTORS IN THE CELLULAR ENVIRONMENT WHICH, IN TURN, COULD ACCOUNT FOR THE OBSERVED ALTERATION OF THE ENERGY METABOLISM OF THE CELLS WOULD BE GLUCOSE AVAILABILITY AND OXYGEN TENSION. THE RESULTS OBTAINED UP TO NOW INDICATE THAT GLUCOSE AVAILABILITY IS NOT RESPONSIBLE FOR THE LOWERING OF THE CELLULAR ATP CONTENT AND THE ENSUING CHANGES. ON THE OTHER HAND, PRELIMINARY EXPERIMENTS INDICATE THAT OXYGEN TENSION MAY BE ONE OF THE MAIN FACTORS DETERMINING CELL MATURATION AND COLLAGEN BIOSYNTHESIS. CONFIRMATION OF THIS FINDING BY FURTHER WORK MAY BE EXPECTED TO HAVE WIDE IMPLICATIONS IN REGARD TO THE MECHANISM OF WOUND HEALING IN MAN. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT
35. EST. FUNDS (In Thousands)	36.

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

Project 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: Edwin M. Bartos, Ph.D.; Hanson H. North, B.S.; James M. Vail, M.S.

Description.

While a great number of clinical studies in man and experimental studies in animals have revealed some of the factors influencing wound healing, the essential determinants of this process continue to elude us: we still do not know what makes fibroblasts proliferate in the early stages of a wound and what, at a later stage, causes these cells to stop dividing and to begin the synthesis of collagen. As long as this ignorance persists we will be essentially powerless to control the course of wound healing in the injured soldier.

The reason for the failure of the previous studies is the great complexity of the clinical situation in man and of the experimental situation in the intact animal. To overcome this difficulty by simplifying the experimental situation, a well defined in vitro culture system is used in this department to study fibroblastic cell populations under normal and simulated trauma conditions.

Progress.

It has been found that under the proper conditions fibroblasts growing in vitro proceed from a logarithmic growth phase characterized by a high rate of cell division and minimal collagen synthesis to a stationary phase characterized by minimal cell division and a high rate of collagen synthesis. The evolution of the fibroblastic cells in this in vitro system was thus shown to parallel the maturation of a wound in the body. In a further search for the determinants of this evolution, it was demonstrated that the arrest of cell division and the initiation of collagen biosynthesis were preceded by a marked decrease of the level of the high energy compound adenosine triphosphate (ATP) in the maturing fibroblasts. The most obvious factors in the cellular environment which, in turn, could account for the observed alteration of the energy metabolism of the cells would be glucose availability and oxygen tension. Extensive investigation of the first of these two alternatives has revealed that glucose availability is not the limiting factor responsible for the lowering of the cellular ATP content and the ensuing changes.

Investigation of the second alternative has necessitated the development of a special oxygen electrode (in conjunction with Instrumentation

Laboratories, Inc.) and associated equipment for the determination of the partial pressure of oxygen (pO_2) in the gas phase and the liquid medium of cultures in the logarithmic and the stationary phases of growth. As shown in the table below, these determinations were carried out after one (T=1) and twenty-four (T=24) hours of incubation. Rates of oxygen consumption were calculated from the difference of pO_2 in the gas phase at T=1 and T=24, the cell density of the cultures, and the time of incubation (23 hrs.).

Growth Phase	Mean Cell Density	pO_2 of gas phase T=1 hr	pO_2 of liquid medium T=1 hr	pO_2 of gas phase T=24 hrs	pO_2 of liquid medium T=24 hrs	O_2 consumption
Logarithmic	67×10^4 cells/ml	151.8 mm Hg	150.0 mm Hg	142.2 mm Hg	128.0 mm Hg	1.63×10^{-10} ml/cell/min
Stationary	616×10^4 cells/ml	150.6 mm Hg	46.5 mm Hg	133.3 mm Hg	7.0 mm Hg	0.38×10^{-10} ml/cell/min

It can be seen that pO_2 of the gas phase declines as a function of the time of incubation, reflecting the consumption of oxygen by the cells. This decline appears to be more pronounced in the stationary culture. However, relative to the number of cells present in this dense culture, the decline is significantly smaller indicating a lower rate of cellular oxygen consumption.

There is also a progressive decline of the pO_2 of the liquid medium of the cultures relative to the pO_2 of the gas phase, indicating that the diffusion of oxygen from the gas phase to the liquid medium lags behind the consumption of oxygen by cells. In the logarithmic culture this decline, negligible after one hour of incubation (T=1), becomes more pronounced twenty-three hours later (T=24). In the stationary culture this decline, quite pronounced at 1 hour, is most dramatic at 24 hours. This is a very significant finding as it suggests that the availability of oxygen in the liquid medium, i.e., the immediate environment of the cells, may indeed be the limiting factor responsible for the lower rate of oxygen consumption of the cells in the stationary phase. This, in turn, would lead to lower ATP levels, arrest of cell division, and synthesis of collagen. Further work aiming to test the validity of this concept is in progress.

Summary and Conclusions.

It has been found that under the proper conditions fibroblasts growing in vitro proceed from a logarithmic growth phase characterized by a high rate of cell division and minimal collagen synthesis to a stationary phase characterized by minimal cell division and a high rate of collagen synthesis. The evolution of the fibroblastic cells in this in vitro system was thus shown to parallel the maturation of a wound in the body. In a further search for the determinants of this process it was demonstrated that the arrest of cell division and the initiation of collagen biosynthesis were preceded by a marked decrease of the oxygen

tension in the environment of the cells and of the high energy compound adenosine triphosphate (ATP) within the cells. The possible determining role of oxygen in initiating the maturation of fibroblasts responsible for wound healing is under investigation.

Publications.

Glinos, A.D. Environmental Feedback Control of Cell Division. In Control of Cellular Growth in Adult Organisms. (H. Teir and T. Rytoma, eds) pp. 41-53, Academic Press, London and New York, 1967.

Vail, J.M., Brown, J.R.C. and Glinos, A.D. Soluble Nucleotide Pools in Log and Stationary Phase L Cell Suspension Cultures. Fed. Proc. 26: 852, 1967.

RESEARCH AND TECHNOLOGY RESUME

1.	2. GOVT. ACCESSION	3. AGENCY ACCESSION	REPORT CONTROL SYMBOL
		DA 0A6428	CSCRD-103

4. DATE OF RESUME	5. KIND OF RESUME	6. SECURITY PPT / AND U	7. REGRADING	8. RELEASE LIMITATION	9. LEVEL OF RESUME
01 07 69	D. CHANGE 01 07 67	U / U	NA	GA	A. WORK UNIT

10a. GOVT. AGENCY NUMBER / CODE: 61145011 3A014501B71P 08 076

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11. TITLE: (U) ANALYSIS OF BEHAVIOR AND OF MEDIATING MECHANISMS-AUTONOMIC AND ELECTROPHYSIOLOGICAL FACTORS

12. SCIENTIFIC OR TECH. AREA: 002400 BIOENGINEERING
012200 STRESS PHYSIOLOGY 012900 PHYSIOLOGY

13. START DATE: 08 63

14. CRIT. COMPL. DATE: NA

15. FUNDING AGENCY: OTHER DA

16. PROCUREMENT METHOD: C. IN-HOUSE

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

18. RESOURCES EST.:

	c. PROFESSIONAL MAN-YEARS	d. FUNDS (In thousands)
PRIOR FY 68	16	280
CURRENT FY 69	16	580

19. GOVT. LAB. INSTALLATION/ACTIVITY

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TEL: 202-576-3457 TYPE: DA

21. TECHNOLOGY UTILIZATION: CLINICAL NEUROLOGY
SURGERY OF TRAUMA

22. COORDINATION: NA

23. NEUROPHYSIOLOGY, NEUROPSYCHIATRY, ANATOMY, AUTONOMIC NERVOUS SYSTEM, CENTRAL NERVOUS SYSTEM, BIOMEDICAL ENGINEERING.

24.

(U) TECH OBJECTIVE - MORPHOLOGICAL AND PHYSIOLOGICAL 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.

(U) APPROACH- DETERMINATION OF CNS PATHWAYS AND TERMINATIONS IN VARIOUS PRIMATES AND SUBPRIMATES BY THE NAUTA TECHNIQUE FOLLOWING APPROPRIATE SURGICAL LESIONS. RECORDING AND ANALYSIS, UTILIZING COMPUTER TECHNIQUES, OF THE CARDIOVASCUALR RESPONSE TO CONTROLLED HEMORRHAGIC SHOCK IN AWAKE CHRONIC MONKEYS. RECORDING AND ANALYSIS OF SINGLE CELL IN THE CNS AND THEIR RELATIONSHIP TO OTHER PHYSIOLOGICAL EVENTS.

(U) PROGRESS - JUL 67 THRU JUN 68 NEW CONCEPTS IN THE EVOLUTION OF CORTICOFUGAL SYSTEMS HAVE BEEN ESTABLISHED. A NEW DATA REDUCTION SYSTEM HAS BEEN DEVELOPED FOR THE HEMORRHAGIC SHOCK STUDIES. DETAILED ANALYSIS OF PHYSIOLOGICAL, ENDOCRINOLOGICAL AND BEHAVIORAL RESPONSES IN THE HEMORRHAGIC SHOCK MODEL ARE CONTINUING. NEW SOURCES OF VISCERAL INPUT TO THE CNS HAVE BEEN FOUND IN THE ADRENAL GLAND. DETAILS OF CNS CONTROL OF BLADDER FUNCTION ARE BEING ESTABLISHED. AUTONOMIC RESPONSES TO INCREASED INTRACRANIAL AND INTRASPINAL PRESSURE HAVE BEEN FOUND. PHYSIOLOGICAL CONCOMITANTS OF CONDITIONED EXPERIMENTAL HYPERTENSION ARE BEING ESTABLISHED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JULY 1967 - 30 JUNE 1968.

26.

27. COMMUNICATIONS SECURITY	28.	29. OSD CODE	30. BUDGET CODE
☐ a. COMSEC OR COMSEC RELATED ☒ b. NOT RELATED		6R	1

31. MISSION OBJECTIVE	32. PARTICIPATION
NA	NA

33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

35. EST. FUNDS (In thousands)	36.

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

Project 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

Investigators.
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Associates: CPT John C. Adkins, MC; CPT John F. Cummings, VC: CPT John J. Dropp, MSC; CPT John C. Hedreen, MC; William Hodos, Ph.D.; Norman Krasnegor, M.A.; CPT Glenn A. Meyer, MC; Noel L. Morlock, M.D.; Akira Niijima, M.D.; 2LT Lloyd D. Partridge, MSC; James M. Petras, Ph.D.; CPT Lawrence A. Plumlee, MC; Andrew T. Prysbylik, EE; Maurice E. T. Swinnen, EE; CPT William G. Troyer, MC.

Description. The general object of this subtask is the analysis of neural mechanisms mediating behavior. The approach which has been followed is twofold: 1) Studies employing the specialized techniques of anatomy, physiology, experimental psychology and endocrinology and 2) Interdisciplinary studies using techniques from several fields simultaneously. Since some aspects of behavior, in its broadest sense, can be studied in greatest detail within a specific discipline, much of the work reported here has been carried out using the classical methods of these disciplines. The questions which have been asked are clearly defined, narrow in scope, and well suited to solution by available techniques. However, many aspects of behavior do not lend themselves to study in this manner. The questions to be answered are not particularly well defined because there is not enough information available to formulate them in an ideal fashion. These 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 being put into interdisciplinary approaches to specific behavioral problems. These pioneer projects besides offering new methods of attack on difficult problems, also serve to strengthen the background of the investigators involved and to aid in a better conceptualization of additional problems to be studied in the analysis of behavior.

Progress.

1. Studies on Efferent Systems.

a. Efferent connections of the neocortex. The main emphasis of this experimental-anatomical study has been on the subcortical projections arising from the precentral and postcentral gyri and the superior and

inferior parietal lobules in the rhesus monkey. Particular attention was paid to the cortical connections with the basal ganglia, diencephalon, and mesencephalon. The longer subcortical connections arising from the precentral and postcentral gyri and parietal lobe have also been traced to the rhombencephon and spinal cord and the results compared. Similar companion experiments on the chimpanzee are also in progress.

b. Efferent connections of the cerebellum. The cerebellar projections to the rh[illegible]ncephalon, mesencephalon, and diencephalon have been studied in the rhesus monkey. Particular emphasis is being given to a comparison of these findings with the corticothalamic projections of the neocortex.

c. Efferent connections of the habenular nuclei. This project yielded 2 lateral habenular lesions showing projections to an area lateral and dorsal to the inter-peduncular nucleus, and extending caudally and dorsally through the decussation of the superior cerebellar peduncle. Two minor habenular lesions without distant projections were also obtained. Difficulty in placing the lesions has slowed progress in this project.

d. Efferent connections to the adrenal gland. Following adrenal medullectomies in neonatal dogs, retrograde cell changes were observed in the zona intermedia of the spinal cord. Chromatolytic cells were most frequently encountered laterally in the zona intermedia in the nucleus intermediolateralis. They were less frequently observed in the nucleus intercalares and least frequently noted paracentrally in the nucleus intermediomedialis. Following left adrenal medullectomy preganglionic cell changes extended ipsilaterally from the fourth thoracic spinal segment to the first lumbar segment. After right adrenal medullectomy chromatolytic cells extended from the third thoracic to the thirteenth thoracic segment.

e. Efferent connections from the superior cervical ganglion. The pineal was stained by Nauta, Fink, and Heimer methods 1, 2, and 3 days following superior cervical ganglionectomy in the rat. No clear-cut degeneration was seen. It was concluded that these particular silver methods are probably not suitable for demonstrating sympathetic axon degeneration.

f. Corticofugal connections with the autonomic nervous system. A comparative study has been made of the corticospinal projections in several species of New World and Old World primates and of several species of carnivores from the families Canidae, Felidae, and Procyonidae. Particular attention has been paid to the projections from the motor cortex in all species and their relationship to autonomic and somatic motor cell groups of the spinal cord. Additional experiments have been performed in the rhesus monkey in order to identify autonomic efferent nuclei at all spinal levels. Autonomic efferent neurons were identified following a variety of thoracic, abdominal, and pelvic nerve lesions.

2. Studies on Afferent Systems.

a. Visual projections in the cat. The optic nerve was cut intracranially in the cat, and allowed survivals of 2, 4, and 7 days. Neither retina nor optic nerve showed any clear-cut degeneration with the Nauta, Fink, and Heimer methods and various modifications thereof. The central nervous system (optic nerve lesion plus some basal forebrain damage in all cases) shows almost no degeneration at 2 days. At 4 days the hypothalamus is unilaterally filled with boutonal degeneration (Heimer, Fink) caused by stria terminalis, preoptic, and other basal forebrain damage. The primary optic projections all degenerate; particularly impressive is the boutonal display in the superficial grey of the superior colliculus, not described in the cat optic projection literature.

b. Visual projections and visual behavior in birds. In order to gain a better understanding of the functioning of the human visual system, we have been studying the visual system of pigeons which is quite similar, but in many ways simpler, than that of primates. We have demonstrated behaviorally and anatomically that nucleus rotundus is the main thalamic relay nucleus for vision in birds. It appears to be comparable to the posterior portion of the mammalian dorsal thalamus which includes nucleus lateralis posterior and the pulvinar. Nucleus rotundus projects to the ectostriatum of the forebrain. We have shown that lesions of the ectostriatum result in behavioral deficits similar to those resulting from lesions of parastriate cortex of inferotemporal cortex of primates. We are continuing a series of studies to determine to what extent the visual discrimination deficits observed following lesions of this system are the result of decreased visual sensitivity. Pigeons have been trained to perform as subjects in various psychophysical procedures. These procedures are quite analogous to those routinely used to determine sensory loss in humans. Threshold elevations have been obtained for minimal separable visual acuity, minimal detectible brightness difference, and minimal detectible difference in flash-rate after lesions of nucleus rotundus. In addition to the tecto-thalamic-telencephalic visual system described above, pigeons also have a thalamo-telencephalic visual system comparable in many ways to the primate geniculo-striate system. We have found that lesions in the dorsal thalamic area which receives the retinal terminations or in its telencephalic projection field result in deficits in brightness and pattern discrimination. Lesions in the thalamic component of this system also result in elevated thresholds in the psychophysical procedures.

c. Auditory projections and auditory behavior in birds. The auditory system of pigeons also bears many similarities to that of humans. Fibers pass from the cochlear nuclei to the nucleus mesencephalicus lateralis, pars dorsalis (MLd) which is the avian homolog of the inferior colliculus. Fibers arising in the MLd pass to the nucleus ovoidalis of the thalamus, which is the avian homolog of the medial geniculate and which in turn projects to a circumscribed zone of the telencephalon (Field L of Rose)

which appears to be comparable in several respects to the auditory cortex of mammals. In several studies now in progress, we are attempting to determine the behavioral significance of these structures by training pigeons to discriminate between different intensities of white noise and between different frequencies of pure tones. Lesions have been made in various parts of the auditory pathway. Although the experiments are not yet complete, the preliminary results suggest that lesions of the MLd result in losses of both intensity and frequency discrimination. However, prolonged post-operative retraining results in recovery of discriminative ability. Other experiments in progress are studying the effects of telencephalic Field L lesions on the discrimination of acoustical patterns. Such discriminations have been shown to be particularly vulnerable to the effects of auditory cortex lesions in cats and monkeys.

d. Forebrain afferents from hindbrain in rodent. Following large lesions in the rat hindbrain, numerous projections to the forebrain have been found. Some of these projections are well known but several new projections have been uncovered. By using a combination of staining techniques, ie., Nauta, Fink and Heimer, an attempt is being made to stain small caliber fiber systems. A comparison between these systems and the serotonin and catecholamine-containing pathways is under study as well as a comparison with the cholinesterase containing pathways.

e. Hypothalamic afferents in the primate. A projection from the medial hypothalamus to nearby medial and lateral hypothalamus has been consistently found. A projection dorsalward to the midline thalamic nuclei is also frequently present. These axons may sometimes extend caudally to rostral central grey, but only in small number. Degeneration in the fornix and septum is usually present also.

f. Physiologic identification of sensory receptors in the adrenal gland. Using neurophysiological techniques of single unit recording, three types of sensory receptors have been identified in the adrenal gland. Mechanoreceptors are present which respond to distensions of the gland capsule and the parenchyma. Baroreceptors were found which faithfully follow the arterial blood pressure level by modulating their firing frequencies. Of most interest are a group of chemosensitive receptors. These receptors, which are tonically active, decrease their firing rate following an increase in the blood level of epinephrine or norepinephrine. Numerous pharmacological studies have been done to demonstrate that this response is markedly decreased and sometimes inhibited by a-blocking agents but unaffected or even facilitated by b-blocking agents. A possibility therefore exists that release of epinephrine and/or norepinephrine is under much more direct neural control than previously thought.

g. Physiologic characteristics of bladder afferents and efferents. The identification and characterization of bladder afferents and efferents using microelectrode techniques has continued. The kinds of physiologic stimuli necessary to activate several distinct types of receptors have been determined. Conduction velocities of afferents in the pelvic plexus and hypogastric nerves have been determined. Work is now concentrated on characterization of bladder efferents. An apparent separation of bladder efferents into tonic and phasic types is under study.

3. Studies on Nervous System Integration.

a. Anatomical basis of spinal autonomic reflexes. Previous anatomical studies have failed to demonstrate a direct bineuronal autonomic reflex arc in the spinal cord. Additional work is being done to establish the relationships between visceral and somatic afferents and the motor efferent cells of the sympathetic and parasympathetic nervous system.

b. Neurophysiological mechanisms of spinal autonomic reflexes. The electrical activity of single neurons of the spinal cord is being studied with intracellular microelectrodes. The project is designed to determine what reflex and integrative functions occur in the autonomic nervous system at the spinal level. Preganglionic sympathetics and interneurons are being studied following antidromic and orthodromic activation procedures.

c. Spinal mechanisms influencing cardiovascular reflexes. This study is directed at establishing the role of intrinsic spinal mechanisms which modify cardiovascular reflexes. By elevating the spinal subarachnoid pressure, a response pattern ensues which closely resembles that seen in the classical Cushing reflex. This reflex is mediated primarily through spinal autonomic pathways, but also involved somato-motor pathways. Neurophysiologic studies have indicated marked changes in excitability in both the autonomic and somatomotor systems. Neuroendocrine responses have also been documented following subarachnoid pressure elevation. The exact mechanisms of the multitude of responses which occur following pressure elevations is under study.

d. Relation between sinus arrythmia and reaction time. Reaction times change during a normally occurring sinus arrythmia. The mechanism of this effect is unknown. Two hypotheses suggest either a central mechanism or a peripheral mechanism via the carotid sinus baroreceptor system. In this study an open-loop analysis is being attempted by eliminating neural control of the heart and artificially pacing it. In this procedure, mean heart rates can be kept constant while the degree of sinus arrythmia can be varied as desired. Reaction times are determined in the monkeys using operant conditioning techniques.

e. Production of chronic hypertension by stress. To determine if chronic hypertension could be elicited by environmental stress, 4 monkeys were placed on an operant training schedule in which all blood pressures below an adjusting criterion evoked a shock to the feet. Low diastolic pressures were punished for 16 or 24 hours a day, causing subjects to receive an average of 1 - 5 foot shocks per hour. Three of the four animals showed elevated diastolic pressures lasting 4-9 weeks. In 2 of these 3 animals, pressures were higher during that part of the day when a discriminative stimulus indicated that low diastolic pressures were punished.

f. Condioned blood pressure elevations. Four monkeys were presented with 10-sec tones which terminated with shocks. The tones were immediately terminated without shock if the animal's diastolic blood pressure rose above a criterion level and remained high for 1 sec. Termination of the tone was followed by a 5-sec time out. Trials began whenever the pressure dropped below the criterion level. All subjects learned the avoidance task, showing diastolic elevations of up to 60 mm of mercury in response to the tones. A linear relationship was seen between the minimum pressure required for avoidance and the pressure achieved. No change in blood pressure accompanied a second stimulus which was never paired with shock. A fifth control monkey was yoked to one of the experimental monkeys and simultaneously received all shocks and tones as determined by the blood pressure of the experimental animal. The yoked control showed no pressure changes to the tones, but normal pressure elevations to shock.

g. Attempts to condition blood pressure decreases. Four monkeys were presented with a sequence of clickers of 10 different frequencies. The highest frequency was always followed after 10 seconds by 5 unavoidable shocks. During other frequencies, the clicker would terminate and produce a 10 second time out if the systolic pressure was lowered below criterion for one second. As a further consequence of lower pressure, the time out was followed by a click of a lower frequency. Failure of the systolic pressure to fall and remain below the criterion for 1 second caused the clicker to increase to gradually higher frequencies until the 10th or shock frequency was reached. An adjusting schedule based on the moneky's performance was used. This schedule made the task easier if the animal failed and more difficult for continuing success. All 4 monkeys showed sudden drops in pressure, but these drops occurred from an elevated baseline or were immediately preceded by pressure rises. Pressure did not fall below the lowest pressures seen among hourly samples during the night. The maximum drops seen were about 25 mm Hg. Sudden drops in pressure were not brought reliably under stimulus control, although the pressure fluctuated more during hours when clickers were presented. It may be concluded that animals on this schedule were taught to fluctuate their blood pressures, sometimes in phase with stimuli spaced 10 seconds apart.

h. Physiologic variables in hemorrhagic shock. The effects of slow hemorrhage as it progresses toward shock are being studied in chronic, extensively instrumented monkeys. The basic aim of this study is to determine the mechanisms and sequences of breakdown of the normal homeostatic reflexes involved in the maintenance of blood pressure. A reproducible model of this form of shock has been established and the natural history of the cardiovascular changes has been determined. Extensive redesign and fabrication of the data collection system have been completed to increase the capability and reliability of the system. In addition to measures of blood pressure, EKG, respiration, venous pressure and EEG, blood flow studies can now be started. New statistical measures of these parameters are also now available because of the changes in the data collection system.

i. Behavioral studies in hemorrhagic shock. Monkeys are trained to avoid electrical stimuli by bar-pressing and required to maintain this behavior during slow blood loss. Additional monkeys have been studied and confirmed the preliminary findings that animals on a behavioral schedule respond differently physiologically to the same physical stimulus (hemorrhage) than naive animals. The mechanisms of these phenomena are under study.

j. Biochemical concomitants of hemorrhagic shock. Blood samples taken prior to and during the hemorrhage procedure are studied for changes in such variables as EPI, NE, 17OH and 17KS, blood sugar, insulin and growth hormone. Some of these variables are also being studied in the operant conditioned animals. A marked rise in EPI occurs coincidently with the second wind phenomenon previously described in the shock studies. Differences have also been found between naive and behaviorally trained animals before and during hemorrhage.

k. Neural control of water balance. A new project has been started to test the relationship between the subcommissural organ (SCO) and water and electrolyte balance in desert rodents and the standard laboratory rodent. Metabolic studies, histology and histochemistry of the SCO are being compared in normal and water deprived animals.

l. Cerebral blood flow changes during sleep. Deep sleep (REM or "dreaming episodes") is accompanied by large cerebral blood flow changes and respiratory depression. Two possible mechanisms for the blood flow changes are an increase of arterial pCO_2 or increased metabolic cerebral metabolism with local CO_2 production. A technique is being developed to measure cerebral flood flow and tissue O_2 tensions. Chronic animals will be respired under neuromuscular block with attendant arterial pCO_2 control and an effort made to determine which of the two possible mechanisms is operating to increase cerebral blood flow.

m. Role of the area Postrema in sleep mechanisms. Numerous clues in the literature suggested that the area Postrema in the medulla might be intimately involved in sleep mechanisms. This region was ablated in chronic cats and monkeys. Subsequent EEG, EMG and direct observations failed to indicate any significant sleep disturbances. Histological studies confirmed adequate area Postrema ablution.

n. Cell proliferation in the adult primate. An autoradiographic study (H_3thymidine) has just started to determine which, if any, cell types of the CNS continue to proliferate in the adult primate. The various cell types synthesizing DNA will be identified, locations noted, and mitotic indices, turnover rates, and generation times determined. Particular attention will be focused on the various glial cells.

4. Technical developments.

a. EMG Integrator. A device to be used with a Grass Polygraph that will integrate the area under the curve of any EMG, EEG, or other periodic signal.

b. Hearing aid. A special hearing aid to be used during conferences. This battery operated device uses operational amplifiers and consumes extremely low power.

c. Transformer. A step-down and step-up transformer to be used during stimulation and recording in physiologic experiments.

d. Logic pattern generator. An instrument that presents a predetermined audio pattern to the experimental animal.

e. Photo-electric pigeon key. A special photo-electric key to be used in the initial training of pigeons.

f. Wave form generator. Developed as a test instrument for exclusive use in the electronics lab as a simulator of biologic parameters.

g. Pulse former supply and diode matrix. A copy of an earlier developed device to be used in conjunction with the INDAR data reduction system.

h. Remote slide projector control. A method to program a slide projector, so as to automatically present visual stimuli to the pigeon.

i. Analog-to-digital converter. A simple A to D converter to enable researcher to record digital data on a standard potentiometric analog recorder.

j. Modification of H.P. counter. A remote control device to program the various functions on a standard H.P. counter.

k. Safety interlock. An instrument that senses power interruptions during unattended periods (e.g., the night) and prevents the power from being re-applied so as to avoid destructive transients.

l. Multiplexer. A six-channel multiplexer to enable blood pressure to be taken from six animals in a time sharing mode.

m. Tape recorder modulator-demodulator. An instrument that permits programing a standard tape recorder by means of recorded audio tones.

n. Pulse formers. Two pulse formers to be used in conjunction with SODECO parallel-entry electro-mechanical counter-printers. This type has two times five decades.

o. Cardiac pacemaker. Two models were built of this device that permits a frequency-modulated pacing of the heart.

p. Pattern generator. A random pattern generator used for the training of pigeons and built entirely with reed relays.

q. Analog computer. An instrument to be used at the electronics lab to simulate various analog functions. It is mainly intended as an electronics design aid.

r. Sequence programmer. A device developed to study the feasibility of using BRS digi-bit cards in sequence programmers, eventually to be used in behavioral studies.

s. Audio generator. A six-channel version of an earlier developed device to present audio stimulation to pigeons.

t. Tape modulator. An instrument to modulate six different levels on an analog tape recorder permitting $2^6 - 1$ or 63 bits of information to be recorded. The device is compatible with BRS digi-bit systems.

Summary and Conclusions.

Previous morphological and physiological studies on neural mediating mechanisms were extended. Several new projects were initiated. Morphological analysis of the central nervous systems of different species 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: 1) peripheral and central control of autonomic reflexes; 2) coding mechanisms of single neurons to various physiologic stimuli; 3) neural mechanisms involved in hormone release. Combined behavioral-anatomical studies have been directed towards establishing functional properties of neuronal aggregates and their relationships to performance. Increased emphasis was placed on elucidating mechanisms of action of the autonomic nervous system and on the anatomical substrate of this system. A new definition of the autonomic nervous system from both an anatomical and a physiological viewpoint is clearly emerging from these studies. The role of this system in homeostatic reflexes and the ability for discrete as well as general actions are new concepts. 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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11. TITLE: (U) ANALYSIS OF BEHAVIOR AND OF MEDIATING MECHANISMS NEUROENDOCRINOLOGICAL FACTORS 09

12. SCIENTIFIC OR TECH. AREA	13. START DATE	14. CRIT. COMPL. DATE	15. FUNDING AGENCY
016200 STRESS PHYSIOLOGY 013400 PSYCOLOGY /INDIVI 005900 ENVIRONMENTAL BIO	08 63	NA	OTHER DA

16. PROCURE. METHOD	17. CONTRACT/GRANT	18. RESOURCES EST.	a. PROFESSIONAL MAN-YEARS	b. FUNDS (In thousands)
C. IN-HOUSE	a. NUMBER NA b. TYPE NA c. DATE NA d. AMOUNT NA	PRIOR FY 68	8	240
		CURRENT FY 69	8	240

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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 PHYSCIAL, AND OF BASIC CONCERN AS AN OBJECTIVE APPROACH IN PSYCHOSOMATIC MEDICINE.

(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.

(U) PROGRESS - JUL 67 THRU JUN 68 RECENT WORK HAS DEMONSTRATED THAT THE ENDOCRINE SYSTEM RESPONDS AS A WHOLE WITH ORGANIZED PATTERNS OF HORMONAL CHANGE WHICH DIFFER IN RELATION TO SPECIFIC PSYCHOLOGICAL AND PHYSICAL STIMULI, INCLUDING FASTING, EXERCISE, DIETARY CHANGES, COLD, HEAT, HYPOXIA, DEHYDRATION,ETC. STUDIES OF SEVERAL PHYSICAL STRESSES, INCLUDING HEAT AND FASTING INDICATE ADRENAL CORTICAL RESPONSES DO NOT OCCUR IF CARE IS TAKEN TO MINIMIZE PSYCHOLOGICAL REACTIONS TO THESE SITUATIONS. THESE EXPERIMENTS SUGGEST SOME MAJOR FALLACIES IN STRESS THEORY WHICH REQUIRE EXTENSIVE REEVALUATION AND RE ISION OF THINKING IN THE STRESS FIELD. SEE DA0A6495, CODE-61130011 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 1967 - 30 JUNE 1968.

26.

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

31. MISSION OBJECTIVE	32. PARTICIPATION
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33. REQUESTING AGENCY	34. SPECIAL EQUIPMENT

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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 role of the central nervous system in the co-ordination of endocrine regulation. Instead of the conventional study of single endocrine systems in isolation, multiple endocrine systems are studied concurrently so that the overall balance between the many interdependent hormones may be investigated. In recent years we have learned that various forms of psychological and physical "stress" elicit broadly organized patterns of hormonal response involving many hormones in addition to those of the adrenal systems. A major goal is to define conclusively these distinctive "overall" hormonal response patterns for various stressful stimuli, including psychological stimuli, cold, heat, hypoxia, fasting, exercise, hemorrhage, dehydration, trauma, infection, and various nutritional changes. A substantial amount of work on the development of new and improved hormone assay procedures, as well as some basic studies of endocrine physiology, have been continued in order to provide the necessary methodological foundation for this stress research program. Some new lines of approach in the study of psychosomatic illnesses and in the study of social factors in psychoendocrine development have been explored during the past year.

Progress.

1. Hormonal Balance in Emotional Stress.

a. Monkey Studies.

(1) Acute Emotional Disturbances. A large series of experiments with a conditioned emotional disturbance (conditioned avoidance) in the monkey have been completed and have shown a complicated but stereotyped phasic pattern of endocrine response involving the 17-hydroxycorticosteroids (17-OHCS), epinephrine, norepinephrine, thyroxine, growth hormone, insulin, testosterone, estrone, estradiol and aldosterone. The results of these experiments, together with an extensive review of the psychoendocrine literature and a presentation of some new concepts suggested by

this work, have been compiled into a monograph which has been submitted for publication as a supplemental issue of Psychosomatic Medicine.

Pilot studies with an adrenalectomized monkey have indicated that while the corticosteroid and epinephrine responses are abolished, the norepinephrine, growth hormone, estrogen, androgen, and insulin responses to conditioned avoidance may still persist. In fact, it appears that the growth hormone response to avoidance may be exaggerated following adrenalectomy. Two additional animals are now under study in this experimental series.

(2) Developmental and Social Factors.

(a) Mother-Infant Interactions. Using operant conditioning techniques, it was learned that infant monkeys worked considerably harder at lever pressing tasks permitting them to regain contact with their mother following separation than did the mother. While adrenal responses were observed on the first separation experience, such responses did not appear on subsequent repetitions, suggesting rapid adaptation to this experience in both mother and infant.

(b) Controlled-Environment Developmental Studies. Two groups of monkeys reared from birth under different conditions, the first remaining with their mothers until three years of age and the second isolated shortly after birth and free of social contact with other monkeys, were studied for their psychoendocrine responsiveness to some standard stimuli after reaching three years of age. No striking differences in mean levels or magnitude of response to restraining chair adaptation were observed between the two groups. It is of interest that considerable individual differences between monkeys in each group were observed, suggesting the importance of either hereditary factors or seemingly minor uncontrolled environmental factors in the determination of psychoendocrine make-up. A third group kept in a gang cage with peer contact from birth on is also scheduled for future study.

(c) Social Hierarchy Studies. An interagency collaborative study, organized by CPT Rose together with Dr. M. Levine of the Human Engineering Laboratory at the Aberdeen Proving Ground, has yielded some interesting leads. Preliminary study of mature monkeys has indicated marked individual differences in psychoendocrine responses to conditioned avoidance may be related to the position of the animal in the social dominance hierarchy.

b. Human Studies. Further analyses of hormonal data from our study of Ft. Dix recruits during basic training in relation to MMPI data have been carried out. Significant correlations between corticosteroid levels and several categories of the MMPI scores indicated that subjects who tended to be outgoing, aggressive, and socially active tended to have lower corticosteroid levels than did subjects who were the opposite, preferring social isolation and less interpersonal involvement. These

findings contrast somewhat with those of previous studies involving quite different life situations, and suggest the need in future studies of psychoendocrine individuality of considering the personality organization in close relation to the current life situation.

2. Hormonal Balance in Physical Stress.

a. Fasting. Although fasting has long been regarded by Selye and others as a "stress," it has been found that little or no adrenal cortical response occurs during fasting if careful measures are taken to minimize psychological reactions to the fasting situation. In the monkey, this was accomplished by shielding the animals from all stimuli normally associated with eating and by providing fruit-flavored but non-nutritive cellulose pellets to reduce the discomfort of an empty gastrointestinal tract during three-day fasting periods. While no corticosteroid response occurred under these conditions, a broad pattern of hormonal response was observed, involving elevations in epinephrine, thyroxine and probably growth hormone levels, with depression in the levels of norepinephrine, insulin, and testosterone.

b. Heat. Although heat has generally been regarded as a "stress" and a pituitary-adrenal stimulus, several pilot experiments in monkeys now indicate that when precautions are taken to minimize possible psychological reactions in the experimental situation, heat may actually inhibit adrenal cortical activity. If the temperature is raised very gradually, one degree per hour for 10 hours, instead of suddenly thrusting the monkey into a hot room, a consistent lowering in corticosteroid excretion has been observed, along with many other hormonal changes including a decrease in norepinephrine, androgen, estrogen, and probably epinephrine levels, with relatively little change in insulin or thyroxine levels. All the observed changes in levels are reversed when the temperature is lowered to normal room temperature (75°).

c. Cold. It does appear that there are bona fide corticosteroid, epinephrine, and norepinephrine responses to cold, even when an effort is made to minimize psychological reaction by a very gradual lowering of environmental temperature. Elevations have also been observed in estrogen and androgen levels during cold in pilot studies with monkeys.

d. Hypoxia. Preliminary experiments in which oxygen levels were lowered from 21% to 14% for 24 hours indicate that corticosteroid, epinephrine, and norepinephrine elevations occur under these conditions. There is some indication that insulin and estrogen levels fall, while growth hormone levels may rise. Physiological adaptation apparently takes place rather rapidly to this experience as the hormonal responses diminish while the hematocrit rises markedly with repetition of these sessions.

e. Nutritional Changes. When monkeys are placed upon a high carbohydrate diet deficient in fat and protein, a broad pattern of hormonal change occurs with elevations in corticosteroid, norepinephrine, and

thyroxine levels and a depression of epinephrine and insulin levels. Preliminary experiments indicate that this response pattern does not occur when adequate protein is supplied, even without fat, but does persist when fat is provided as long as protein is withheld.

f. Dehydration. Pilot experiments indicate that reducing water intake from 300 to 400 cc. down to 60 cc. per day elicits relatively little change in corticosteroid and epinephrine levels while rather marked decreases in estrogen, androgen, norepinephrine, and aldosterone excretion were observed.

3. Hormonal Balance in Medical Illnesses.

An attempt was made to measure broad hormonal patterns in some medical patients during the past year, with the view that disorders in hormone balance related to psychological or other factors may play a pathogenetic role in some medical illnesses. Pilot studies of two patients with rheumatoid arthritis and two diabetics were of value primarily in indicating some complicating experimental variables such as medication, pain secondary to illness, and physical activity which will require careful consideration in the future exploration of this approach. Work on this approach has been temporarily suspended until a medical officer can be assigned full time to this project.

4. Biochemical Methodology.

Special progress has been made this past year in several areas. In addition to our established radioimmunoassays for plasma insulin and growth hormone determinations, new radioimmunoassays for the measurement of plasma luteinizing hormone, plasma thyrotropin, and plasma glucagon levels have been developed and validated from an immunochemical standpoint. At present these methods are being evaluated from a physiological standpoint.

The application of protein-binding-isotope methods of steroid measurement has also been extended this year. An improved, highly sensitive method for plasma cortisol measurement, requiring only 0.1 ml. of plasma, has been developed. A new method for free urinary cortisol measurement has been set up, validated, and is now being compared with our established total 17-OHCS measurement in stress studies. A protein-binding method for plasma testosterone measurement has been developed and is currently being validated. The development of a semiautomated urinary catecholamine procedure has progressed steadily and is near completion at present.

5. Endocrine Physiology.

The broad hormonal response pattern to ACTH and gonadotrophin injection has been completed. These studies have been further evaluated in an adrenalectomized monkey so that we now have knowledge of the relative

proportions of steroid hormones coming from adrenal versus testicular sources in the monkey. The effects of selectively increasing the levels of one hormone such as cortisol, thyroxine, or testosterone by injection upon the "overall" balance of the many other hormones is also currently under study to provide information of value in the interpretation of our "stress" experiments.

Summary and Conclusions.

Two major concepts are emerging from our studies.

First, our studies indicate that the "stress" field must begin to move away from a preoccupation with the adrenal systems alone to a consideration of the many other hormonal changes which appear to be organized distinctively in a broad, overall manner for different psychological and physical stimuli. Further study of broad patterns of hormonal change seems likely to uncover some important basic principles of physiological co-ordination or integration.

Secondly, it appears that the entire field of "stress" theory is thrown into confusion by the new findings that the adrenal response to many "physical" stimuli is probably spurious and actually related to contaminating psychological reactions involving emotional arousal, discomfort, or pain rather than the physical stimulus itself. It is imperative, therefore, that a painstaking re-evaluation of the many different "physical stresses" be carried out with close attention to minimizing and assessing possible interfering psychological factors.

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PROJECT 3A014501B71P
BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09
Radiobiology

RESEARCH AND TECHNOLOGY RESUME

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(U) TECH OBJECTIVE - QUANTITATIVE MEASUREMENTS OF THE CONSEQUENCES OF EXPOSURE TO IONIZING RADIATION IN CELLULAR, SUBMAMMALIAN AND MAMMALIAN SYSTEMS.

(U) APPROACH- EFFECTS OF RADIATION AND RADIATION MODIFIERS ON DNA AND INFLUENCE OF ALTERATIONS IN CELL METABOLISM ON RADIATION SURVIVAL ARE BEING INVESTIGATED IN BACTERIA. ELECTRON SPIN RESONANCE SPECTROMETRY IS USED TO DEFINE FREE RADICAL PRODUCTION AND THE TIME LENGTH OF ACTION OF RADICALS WITHIN THE BIOLOGICAL MEDIUM. CHANGES IN GENETIC MATERIAL WITHIN MAMMALIAN CELL LINES AS EVIDENCED BY CHROMOSOMAL BREAKS AND TRANSLOCATIONS ARE QUANTITATED WITH BIOCHEMICAL CELLULAR CONSTITUENTS AFTER RADIATION. INFLUENCE OF STRESSES SUCH AS TRAUMA AND BURNS COMBINED WITH RADIATION ARE EVALUATED IN RELATION TO DOSE RESPONSE.

(U) PROGRESS - JUL 67 THRU JUN 68 ANALYSIS OF DNA STRAND BREAKS IN IRRADIATED E. COLI CONTINUE. STUDIES OF RADIOBIOLOGICAL ROLES OF INTERSTRAND CROSSLINKS IN DNA WERE INITIATED. ESR-RADIATION DOSIMETRY AND ESR-RADIATION SURVIVAL STUDIES ARE CONTINUING. EFFECTS OF RADIATION EXPOSURE ON BEHAVIOR PATTERNS ARE BEING STUDIED IN INSECTS AND HIGHER ORGANISMS. STUDIES ON RADIATION EFFECTS ON WOUND HEALING HAVE BEEN EXTENDED. HISTOLOGICAL AND HISTOCHEMICAL RESPONSES OF EXTERIORIZED RODENT SMALL INTESTINE TO X-IRRADIATION ARE BEING INVESTIGATED. FOR TECHNICAL REPORTS, SEE WALTER REED ARMY INSTITUTE OF RESEARCH ANNUAL PROGRESS REPORT, 1 JUL 1967 - 30 JUN 1968.

26.

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Project 3A014501B71P, BASIC RESEARCH IN SUPPORT OF MILITARY MEDICINE

Task 09, Radiobiology

Work Unit 015, Mechanisms

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Description.

Comprehensive knowledge of the biochemical and physiological basis of radiation injury in humans and other mammals is necessary for the recognition and understanding of the clinical and pathological manifestations of radiation injury, and for the development of effective prophylactic and therapeutic measures. Therefore, the objective of this work unit is to define the biochemical and physiological mechanisms by which ionizing radiation affects living organisms, submammalian and mammalian,at the levels of physico-chemical, molecular and cellular, tissues, organs, and whole organisms. Research efforts under this work unit include:

1. Physico-chemical studies of radiation injury.

Electron spin resonance techniques are being used in the detection, measurement, and evaluation of physico-chemical parameters of radiation injury. Included in these studies are investigations of effects of various radiation response modifiers on metabolism in tissue from normal and irradiated animals.

2. Molecular and cellular biology of radiation injury.

The objective of this work is to identify and measure specific kinds of ionizing radiation damage to biomacromolecules within cells, and to determine the biochemical mechanisms involved in the induction and repair of specific cellular lesions.

3. Submammalian species response to ionizing radiation.

Studies on the volitional activity of various submammalian species are designed to determine the influence of radiation and radiation modifiers on gross activity.

4. Mammalian tissue, organ, and whole organism studies.

Immediate and long-term changes in functional integrity, chemical consistency, and genetic constitution of whole mammals after exposure to ionizing radiation are being investigated. These studies include:

a. Combined injury studies. The relationship of radiation injury, particularly bone marrow depression and its sequelae, to trauma.

b. Gastrointestinal studies. The histological and histochemical alterations attendant to X-irradiation of the exteriorized small bowel in rats and the effect of radiation-induced destruction of the anatomic integrity of the small intestinal mucosal cell on gastrointestional adsorption of ^{59}Fe.

c. Mortality after mixed radiation (neutron-gamma) exposure. Lethality, comparative pathology, and hematology are being studied in germfree mice after exposure to mixed radiation (fast and thermal neutrons and gamma radiation) in the Walter Reed Research Reactor.

Progress.

1. Physico-chemical studies of radiation injury. (LTC Swartz, Dr. Copeland, Dr. Heller, Mr. Richardson).

a. Tissue studies. Studies of the relationships between various physiological and environmental parameters and electron spin resonances (ESR) in unirradiated tissues (WRAIR Annual Progress Report, 1966-67) have been continued. These studies were undertaken to determine what factors might affect the "background" tissue ESR signals. The signals due to irradiation are superimposed on these "natural" signals and at very low doses the two types may be of comparable magnitude. The results of these studies suggested that the natural ESR signals of tissue may also be of value in basic biological and clinical investigations. For example, the changes in ESR spectra of tissues have been found by some authors to precede histological and (detectable) biochemical changes in carcinogenesis. With this in mind the initial studies have been extended to permit evaluation of the possible applications of electron spin resonance spectrometry to clinical investigation. The results so far have been very promising.

1) Comparison of frozen and unfrozen tissue ESR spectra. It is necessary to study actively metabolizing liver tissue if information concerning ESR spectra in normal living states is to be obtained. This approach is relatively tedious and time-consuming. Quick freezing of tissue stops tissue radical reactions and permits the observation at leisure of large numbers of samples obtained from the same experiment at the same time. The sensitivity of the ESR spectrometer is also much greater for frozen samples by a factor of about 100. In order to take advantage of the greater sensitivity and experimental ease resulting in

the use of frozen samples, however, we must first show that the signals seen in the frozen samples have a definite relationship to those seen in the living slices. Experimentally we are doing this by observing liver slices in the ESR spectrometer and then freezing these same slices and re-examining them in the frozen state. The liver slices were prepared from animals that were subjected to different treatments (diet restriction, thyroid hormone, etc). Experiments were also done on liver slices that were modified in vitro (heat, cyanide, etc). Two types of tissue ESR signals have been identified in these studies. The first, located within the free radical resonance area, appears to have similar intensities in both fresh and frozen liver slices. The second type, located both at the free radical area and at many other places in the spectrum, is seen mainly in the frozen samples and does not correspond closely to the signals seen in the unfrozen specimens.

2) Effects of physiological parameters on frozen liver ESR signals. As a result of the implied relationship between the signals seen in fresh and frozen liver slices, a series of experiments have been begun using frozen liver samples exclusively. These include studies of the effects of fasting, an exclusively glucose diet, thyroid, bile duct ligation, beta-mercaptoethylamine, and phenobarbital. These effects are being followed as functions of time and level of administration or manipulation. To date changes have been seen with each of these treatments except thyroxine and phenobarbital. Quantitative data of these effects are not yet available.

3) Post mortem changes in liver. The spectra of frozen liver samples obtained serially after death show characteristic time and temperature dependent changes. These changes have now been studied in a number of animal species (mice, rats, sheep, pigs, monkeys, and rabbits) at 0, 20, and 37°. The reproducibility of these changes suggests their use as a method of determining time of death in forensic medicine. In cooperation with the AFIP we are now receiving human liver samples from autopsies and will attempt to correlate the spectral changes observed in these tissues with the known time of death.

4) Organ viability determination. The nature of the tissue ESR signals, their proposed origin in metabolic processes, and the above changes noted after death all suggest that these signals may provide a means of evaluating viability of organs prior to transplantation. In collaboration with Dr. Starzl's group at the University of Colorado an extensive study is now in progress. Liver transplants are being performed on a series of dogs. Donor livers are preserved for variable time under several conditions before transplantation into recipients. Biopsies of these livers are obtained just prior to transplantation and these are frozen and shipped to WRAIR for ESR analysis. We will try to relate the ESR changes with the organ preservation methods and the long term success of the transplants. This technique may supply information prior to possible transplantation that will permit an accurate estimate of the viability of the organ.

5) Reticulocytosis. Frozen red blood cells normally have a very small ESR signal. Our studies have demonstrated that this signal increases greatly in intensity and changes in shape following chronic hemolysis. This effect paralleled the degree of reticulocytosis and appeared to be related to the hematopoietic process. The younger reticulocytes produced the more intense ESR signals. This method may lead to specific information on the process of hemoglobin synthesis.

b. In vivo dosimetry. Studies of the usefulness of radiation-induced long-lived ESR resonances in biological tissues for in vivo dosimetry have been summarized and are in press, (Health Physics Journal). Many tissues were found to have persistent ESR signals following irradiation, expecially hard tissues such as bone and teeth. The latter gave a linear response to radiation doses from less than a 100 to over 40,000 rads. Because of the obvious problems in obtaining teeth as specimens from surviving radiation victims, other tissues have also been investigated. The signal in hair is modified by water and is therefore unreliable. Red blood cells give a characteristic signal but this can be detected only at doses greater than 25,000 rads. Preliminary work with fingernails suggests that this tissue may provide the desired sensitivity and stability of signal and it is easily accessible. Currently our best dosimetry is with teeth, which provide dose estimation of ± 50 rads in the range 100 - 1000 rads.

c. Oxygen effect, freezing and protection. In a previous WRAIR report we hypothesized that several damaging treatments (radiation, hyperbaric oxygen, lyophilization, drying and freezing) contained a common mode of damage, namely the induction or release of free radicals capable of reacting with oxygen. If correct this hypothesis would help shed light on the nature of the damage caused by all of these processes, including the clinically important field of oxygen toxicity. The hypothesis predicts that there should be an oxygen effect in freezing damage. The literature on freezing damage does not usually consider a possible role of oxygen in freezing damage. We have performed initial studies in bacteria which demonstrated the production of free radicals by freezing, and the amount of free radicals production was increased if oxygen was present. These findings were in accord with the hypothesis, but we did not find the predicted decrease in survival rate. However, by adding the stress of radiation the predicted biological effect has now been found. In the critical experiment, bacteria were frozen in nitrogen or oxygen saturated solutions. These solutions were then thawed, the oxygen was removed by nitrogen purging, and the bacterial suspensions were irradiated. The samples frozen from the oxygen containing solutions had a lower radiation resistance than those frozen in the oxygen free solutions. This indicates that freezing in the presence of oxygen does cause sublethal damage that can be brought out by adding a second stress. E. coli with a good damage repairing system (strain B/r) were able to handle these two damages better than a strain (B_{s-1}) with a poor damage repairing system. As expected from radiation biology and this hypothesis, the radioprotective drug MEA reduced this combined damage effect.

d. Nature of difference between radiation sensitive (B_{s-1}) and radiation resistant strain (B/r) E. coli. Although these two strains of E. coli differ tremendously in their radiation resistance, their qualitative and quantitative responses were similar with respect to free radical formation. The effects of O_2, N_2 and MEA on these radicals are also the same in both strains. This finding is consistent with the hypothesis that the difference between these strains is due to differences in their ability to repair damage, rather than to differences in the initial amount or kind of damage inflicted. In bacteria with the ability to repair potentially lethal damage, a slower rate of growth should allow more time for the repair process to occur, and, therefore, an enhancement of radiation survival might be expected. To test this hypothesis, E. coli strains B/r and B_{s-1} were irradiated and then grown on blood agar (fast growth) and minimal agar (slow growth) media. Irradiated strain B/r showed no difference in survival on the two media, while B_{s-1} showed increased survival on the minimal medium. A possible interpretation of this unexpected result is: (a) B/r can repair radiation damage but does it so efficiently that even on fast growth media repair was completed before the critical period (presumably cell division), (b) B_{s-1} has only a limited capacity for repair which is not efficient enough to be fully expressed on the fast growth medium, but which can enhance survival if growth is slowed down by plating on minimal media.

2. Molecular and cellular biology of radiation injury. (LTC Ginsberg, CPT Hawkins).

Experimental techniques and preliminary findings were described under "Basic Research in Support of Military Medicine", Task 09, Radiobiology, Work Unit 015, Mechanisms, WRAIR Annual Progress Report (1 July 1966 - 30 June 1967). The initial studies were confined to one strain, 15TAU-bar, of the bacterium E. coli. Experimental work has been extended to two additional strains; strain B_{s-1}, a very radiosensitive mutant of E. coli, and strain B/r, a highly radioresistant mutant.

In both logarithmic and stationary growth phases, strain 15TAU-bar exhibits survival curves having slightly greater slopes at high (above 20 Krad) than at lower doses of gamma radiation, while strains B_{s-1} and B/r exhibit nearly exponential gamma radiation survival curves (the initial and final slopes being similar). The D_{37}'s of the respective cultures irradiated in phosphate buffer with and without MEA (0.04 M) are shown in Table I.

Strain B_{s-1} is unique among the three strains tested in that the D_{37} is the same for logarithmic- and stationary-phase cultures. It has been proposed that the difference in radiosensitivity between logarithmic- and stationary-phase cells of a particular strain is due to differences in ability to repair radiation damage rather than to differences in the kind or amount of damage. It is believed that mechanisms for repair of radiation damage are highly inefficient or entirely absent in strain B_{s-1}, and the observation that B_{s-1} cultures in different growth phases do

TABLE I

D_{37}* FOR *E. COLI* STRAINS IN DIFFERENT GROWTH PHASES

Strain	Irradiated in Buffer			Irradiated in 0.04 M MEA		
	logarithmic	stationary	-AA**	logarithmic	stationary	-AA**
B_{s-1}	1.7	1.7	--	3.7***	3.8***	--
B/r	7.1	12.5	--	27.1	43.3	--
15TAU-bar	4.4	13	13	16.0	40	43.6

*In kilorads, measured directly from survival data plotted on semi-logarithmic scales.

**Logarithmic-phase culture grown for two hours without required amino acids.

***The difference in D_{37} between logarithmic- and stationary-phase cultures of strain B_{s-1} irradiated in MEA, though small, is significant, as indicated by the continued divergence of the survival curves at higher doses.

not show differences in radiation sensitivity is consistent with this postulate.

Single-strand breaks produced in vivo in DNA by 20 Krad of Cobalt-60 gamma irradiation were measured in all three strains, in both growth phases, and in the presence and absence of a chemical radioprotectant, beta-mercaptoethylamine (MEA).

The amount of 20 Krad radiation damage (measured as single-strand breaks) in the DNA was the same in all three strains and was independent of growth phase. Although MEA protected against killing in all strains in both growth phases, it protected against single-strand breakage of DNA only in stationary-phase cultures in all three strains. Several possible explanations for this observation are possible: For example; (1) in order to protect against single-strand breaks, the MEA must penetrate the cell envelope, which it can do in stationary- but not logarithmic-phase cells; or (2) MEA can penetrate these envelopes equally well, but in order to protect against single-strand breaks, the MEA must form a chemical bond somewhere on the DNA molecule, which it can do with DNA from stationary-phase cells only. Present experiments cannot distinguish among such possibilities.

It has been proposed by other workers that the greater radiosensitivity of strain B_{s-1} compared to B/r is due to the inability of B_{s-1} to repair single-strand breaks. Double-strand breaks are considered unrepairable and equally lethal in both strains. Strain B/r can repair single-strand breaks; therefore, single-strand breaks are not lethal for B/r. Strain B_{s-1} cannot repair single-strand breaks; therefore, such breaks are lethal in B_{s-1}. Results of experiments described here are not consistent with this interpretation. That is, if the difference in radiosensitivity between B/r and B_{s-1} were a matter only of different abilities to repair single-strand breaks, the survival curve of stationary phase B_{s-1} irradiated in MEA should show at least the same radioresistance as for stationary phase B/r irradiated in buffer. This would be expected because MEA protects against single-strand breaks in strain B_{s-1}. However, this is not the case, as shown in Table I. The D_{37} for stationary-phase B/r irradiated in buffer is three times that for stationary-phase B_{s-1} irradiated in MEA.

Furthermore, the following results show that there is no systematic correlation between single-strand breaks and killing of E. coli: Strain 15TAU-bar was grown to active logarithmic phase and then deprived of required amino acids for two hours. This treatment results in a change in metabolic state of the culture so that it becomes physiologically and radiobiologically indistinguishable from stationary phase. Not only are the UV and gamma-ray survival curves of the amino-acid starved logarithmic-phase culture identical to those of normal stationary-phase cultures, but the spectrum of lethal gamma-ray damage (as measured by survival) prevented by MEA is identical. However, MEA does not protect gamma-ray induced single-strand breaks in the starved culture. In this respect,

the culture retains its logarithmic-phase character. This result is consistent with other data if one assumes that the change in metabolic state during amino acid starvation does not significantly alter the characteristic of the cell envelope that makes it act as a barrier to penetration by MEA, or that the change does not alter the chemical bonding property (with respect to MEA) of DNA. In any event, it seems for the present that results pertaining to effects of MEA on gamma-ray induced strand breakage and those pertaining to effects of MEA on radiation survival must be interpreted independently.

Thus, without regard to radicbiological significance of strand breaks, it is concluded that, in order to protect against strand breakage, MEA must penetrate the cell envelope(s). Furthermore, it is concluded that MEA can penetrate the stationary-phase cells and, if required for protection, can form an association with the DNA. It is also concluded that MEA does not protect against strand breaks in logarithmic-phase cells, because either the MEA cannot penetrate the logarithmic-phase cell envelope, or, if it can penetrate, it cannot form the necessary chemical association with DNA.

Similar studies of double-strand breaks in DNA from irradiated cells were initiated. Acceptably reproducible results have not been obtained despite intensive efforts to overcome the technical difficulties of gentle isolation of DNA at or near neutral pH. Work has also been started to study induced crosslinks between complementary DNA strands in irradiated bacteria. Such crosslinks may be of more apparent radiobiological significance than are the single-strand breaks.

3. Submammalian species response to ionizing radiation. (Dr. Krebs, Mrs. McLaughlin).

Field commanders currently utilize probably unreliable data concerning the degree and time course of mental and physical incapacitation in humans exposed to sub-lethal, lethal, and supra-lethal doses of radiation. Better estimates may result from studies of radiation effects on work capacity and performance quality in laboratory animals. Such studies using primates are in progress at other laboratories. A limited study of behavioral effects of radiation in arthropods has been conducted in this laboratory. Ants are practical subjects for such studies because they exhibit highly ordered wo.k and social behavior patterns.

California Harvester Ants, Pogonomyrmex californicus, were exposed to doses of Co-60 gamma radiation up to 156,000 rads. Performance ability (especially digging and tunneling) declined as a function of dose. After irradiation, the performance changes resembled those reported for mammals (there was an initial depression which was followed by a transitory rise). A different response pattern was observed in alarm and defense behavior: The readiness of the ants to show alarm and defense activity (i.e., a characteristic attack posture and an immediate readiness to damage and dismember intruder ants) was unimpaired at all administered doses. How-

ever, there was a progressive and dose-dependent decline in ability to respond, as indicated by the increasing times required to annihilate intruding ants. Other physical abilities were also depressed, and at higher doses the ants began to confuse the usually well ordered sequences of work activity.

Although ants and mammals differ anatomically, physiologically, and radiobiologically, they show many similarities in work-behavior patterns and in the effects of radiation on these patterns. These studies raise interesting questions concerning the mechanisms underlying the changes in behavior after irradiation as well as the reasons for similarity in responses among different species.

This study model may prove useful in further investigations of the behavioral effects of supra-lethal irradiation. Continuation of these studies at the WRAIR is not planned.

4. Mammalian tissues, organ and whole organism studies.

a. Combined injury studies. (CPT Donati, LTC Mahin, LTC Stromberg, CPT Frank, Mrs. McLaughlin, Mrs. Davis).

1) Radiation and surgical wounding. The obvious military significance of the problem of combined radiation and traumatic injury provided the impetus to evaluate the effects of irradiation on open wound contrac' The initial approach was to study the relationship between time of irradiation, time of wounding, and rate of wound contraction in the rat. A dose of gamma radiation in the mid-lethal range (675 rads) was utilized at various time periods prior to and following wounding. Generalized bone marrow depression characterized by pancytopenia, hemorrhagic diathesis, and diminished resistance to infection was a major effect of whole body irradiation in the dose utilized. Animals wounded prior to irradiation demonstrated a minimal transient cessation of wound contracture; those wounded following irradiation demonstrated an immediate and much greater interference with wound contracture, particularly in the group wounded four days following irradiation.

This delay in wound contracture was accompanied by an alteration in the histologic characteristics of the wound as determined by light microscopy. In the non-irradiated animal, strands of fibrin interspersed with acute inflammatory cells, mainly granulocytes, a few lymphocytes and numerous extravasated erythrocytes appear within twelve hours of wounding. Large spindle-shaped cells, fibroblasts, presumably synthesizing collagen, as well as neutrophils and large monocytes, begin to infiltrate the wound within 24 hours. The typical appearance of granulation tissue is established by the sixth day.

The wounds of rats wounded four days following exposure to 800 R X-irradiation manifested an alteration in the time sequence of histologic changes. There was a more marked extravasation of red blood cells and a

delay in the inflammatory response. There was a diminution in number of fibroblasts as well as a delay in appearance of these tissue elements and the subsequent formation of collagen.

Several possible explanations were considered for the alterations in wound healing in the irradiated animal. A delay in affixation of the skin edge of the wound to the underlying tissue is one possibility. By the time the skin edge is attached, the wound is larger and therefore closure is delayed. This would suggest the existence of a fundamental defect in the relationship of the wound edge to the wound base. Such an alteration could be due to a defect in fibroblastic function in the granulation bed secondary either to the deleterious effects of infection or the catabolic effects of irradiation. Alternatively, this defect could reflect post-irradiation alterations in bone marrow function. Fibroblastic function may depend upon a competent bone marrow in one of two ways: The bone marrow may be the direct source of fibroblastic precursors, as has recently been suggested, or fibroblasts in the wound may require support by the marrow. The severe bone marrow depression which follows irradiation would then be associated with depression of fibroblastic function. Such a defect could result in a delay in adherence of the wound edge to the granulation tissue bed.

Several of these possibilities have been explored. The possible effects of infection on the rate of wound contraction have been studied. Wound contraction following irradiation was assessed in rats with the concurrent administration of antimicrobials. The administration of tetracycline, chloramphenicol, or streptomycin diminished the 30 day mortality but did not alter significantly the wound healing pattern in the irradiated wounded rat. The characterization and distribution of bacteria revealed increased bacterial contamination of greater distribution in the irradiated wounded rat. In addition, an uncontrolled and variable bacterial contamination was precluded by determining the effect of irradiation on wound repair in the germfree animal. The wound healing pattern of the germfree irradiated animal did not differ significantly from that of the conventionalized animal. However, the 30 day survival was markedly increased in the germfree animal. These data are interpreted as strong evidence, suggesting that the deleterious effects of infection do not play a significant role in the production of the altered wound healing pattern in the irradiated rat. However, these data confirm the previously reported beneficial effect of antimicrobial treatment (and control of infection) and the germfree state on the mortality following irradiation.

The possibility that the diminished rate of wound contracture observed in the irradiated rat may be secondary to post-irradiation alterations in bone marrow function was also assessed. Wound contraction was measured in irradiated bone-marrow-shielded rats. The bone marrow shielding induced a marked reversion toward normal in the wound contracture pattern. The increased mortality attendant to the addition of wounding to radiation injury was also reduced, in part. In addition, histologic observations revealed a reversion toward the normal histologic

pattern in the wound of the irradiated bone-marrow-shielded rat.

Prior therapy with three known radioprotective drugs (MEA, serotonin, and WR 2721 C) produced a survival rate in the treated, wounded, irradiated rats very similar to that of the non-wounded irradiated rats. To a variable degree, these radioprotective agents also produced a reversion of the wound contracture patterns toward normal.

Furthermore, studies were carried out in which the irradiated rats were transfused with marrow from litter mates and subsequently wounded. This study produced results similar to those obtained from the marrow shielding and radioprotective experiments.

In view of the foregoing, we have concluded that the integrity of the marrow is necessary for normal wound healing. Furthermore, we conclude that the alteration in wound healing attendant to radiation is the result, in part, of the marrow damage which follows radiation injury. The exact cell types and mechanisms involved in this intimate relationship between the marrow and wound healing remain to be explored.

2) Radiation and gravitational stress. (Dr. Krebs, Mrs. McLaughlin). Studies on the effects of combined injury - radiation and gravitational stress - on C-57 mice were continued. Besides centrifuging the mice during X-irradiation in a position perpendicular to the radius of the centrifuge, mice were also centrifuged and irradiated with the head in the center of the centrifuge and, vice versa, with the pelvis in the center and the head outside. In all three cases different results were obtained. The data are currently being evaluated and interpreted.

b. Gastrointestinal radiation injury. (CPT Donati, Dr. Jervis, Miss Berman, Mrs. Davis, COL Sprinz). Radiation injury to the small intestine is characterized by alterations in motility, function, and morphology. The purpose of this project is to characterize more exactly the response of the small intestine to ionizing radiation and to elucidate the mechanisms responsible for these responses.

We have performed histological and histochemical studies of the response in the small intestine to 2000 R X-irradiation delivered to the exteriorized small intestine of the rat, the abdomen of the rat (with the body shielded), or the whole body of the rat. The results demonstrated a shortening of the small intestine which was time dependent. In addition, when the dose was applied to the whole body or to the abdomen only, the characteristic lesion of acute intestinal radiation damage was seen but without generalized epithelial denudation of the mucosa. The same dose, when delivered to the exteriorized gut, produced patchy lesions with areas similar to those observed in rats receiving irradiation to the whole body or to the abdomen only, interspersed with apparently intact areas. There were indications that the latter areas were protected by anoxia induced by local interference with blood supply.

The histochemical findings are presented in detail in the report of experimental pathology.

The effect of this radiation-induced small intestinal mucosal damage on the gastrointestinal absorption of radioiron was also assessed. Details of procedure are presented in WRAIR Annual Report, FY 1967. Through the fourth day post-irradiation, the gastrointestinal absorption of radioiron was only slightly elevated in the irradiated animal; however, on the fourth day absorption was markedly elevated and returned towards normal by the fifth day post-irradiation. These results suggested that the small intestine was capable of absorbing iron even when the integrity of the mucosal cell as determined by histologic criteria was severely compromised due to radiation injury. Subsequent studies demonstrated that an injected intravenous dose of radioiron does not "leak" out into the intestinal lumen through the severely damaged mucosal cell and suggest that this process is unidirectional. In addition, gross radioautographs of the small intestine following irradiation of the exteriorized gut and an oral dose of radioiron showed that the undamaged areas of the small intestine concentrate radioiron suggesting that these areas are responsible for augmented gastrointestinal iron absorption. Further studies are contemplated to determine the rate of gastrointestinal absorption of various metals following radiation injury, and the possible detrimental effects and the role which altered absorption might play in the radiation syndrome.

The morphologic studies, in addition to demonstrating a discontinuous, interrupted response of the small intestine to X-irradiation, also demonstrated overcompensation or greater-than-normal regeneration in the relatively uninjured areas. This phenomenon has been described in the response to radiation injury in other tissues and has been characterized as an "overshoot" phenomenon. Histologically, the characteristics are that the regenerating villus is longer, the cells more numerous, and the rate of renewal hypernormal. The mechanism responsible for the "overshoot" during the repair phase following small intestinal irradiation is unclear. It was therefore elected to study this mechanism. The initial approach was an attempt to determine whether a transmissible plasma factor was responsible for the regeneration of the villus epithelium following radiation injury. Plasma was harvested from Wistar rats five days following the exposure of the exteriorized small intestine to 2000 R X-radiation. This plasma was frozen until used. Subsequently, aliquots of plasma from irradiated or normal rats were injected in various time patterns into recipient mice. The mice were then given a pulse label of tritiated thymidine and sacrificed one hour, 24, or 48 hours later. The small intestine was removed and prepared for radioautography. The radioautographs were evaluated for activity by means of crypt cell grain counts and the level to which the radioactivity progressed up the villus. The results of this initial study revealed a great deal of variability and overlap between the control and experimental groups, and no conclusions could be reached. The great variability was considered due to variability in bacterial flora of the mice used, but this was not proved.

In order to eliminate this variable, a similar study was undertaken utilizing germfree mice. The results of this study were also inconclusive but suggested a stimulatory effect produced by plasma obtained from rats five days following irradiation of the exteriorized small intestine.

At this point, we believed that exposure to this plasma for a longer period of time, (more or less continuous exposure), might demonstrate a clearcut effect. Therefore, inbred Lewis strain rats were parabiosed. Following recovery from the operative procedure, the small intestine of one member of each parabiont pair was exteriorized and exposed to 2000 R X-radiation. Five days thereafter a pulse label of tritiated thymidine was given, the parabionts were killed, and the small intestine of the non-irradiated member of each parabiont pair was prepared for radioautography. The results of preliminary experiments utilizing parabiotic rats suggest that no transmissible humoral factor can be demonstrated under these specific circumstances.

c. Mortality in gnotobiotic and conventional mice after mixed radiation (neutron-gamma) exposure. (Mrs. McLaughlin, Dr. Krebs, Dr. Jervis, COL Sprinz, Mrs. Davis). Infection is well recognized as a complication in the post-irradiation syndrome of humans as well as experimental animals. It unquestionably contributes to, and often is the major cause of, post-irradiation death. Because it is difficult to separate radiation-induced changes as such from those due to the variable effects of uncontrolled laboratory infections (clinical and sub-clinical), the bacteria-free animal has become an extremely useful tool for exploring the relation between infection and radiation, and for basic studies of mechanisms of radiation damage. Previous exhaustive studies (anatomical, physiological, biochemical, hematologic, and nutritional) have described only minor differences between germfree animals and conventional controls.

Reyniers of Notre Dame first reported in 1950 that several germfree rats had survived doses of radiation which were fatal for ordinary rats. Since then, numerous studies have demonstrated differences in mortality and survival time, as well as hematologic and histologic characteristics, and have confirmed that bacteria-free animals are remarkably resistant to X-irradiation.

Up to the present time, all of the studies have dealt with the influence of the bacteria-free state in animals exposed to X-rays. Mortality in nongermfree mice after acute neutron irradiation has been closely correlated in time with the development of generalized infection. Treatment with antibiotics reduces mortality after neutron irradiation, although not to the same degree as after X-irradiation at doses producing "marrow" injury. It is generally felt that infection plays a less important role in death after neutron irradiation than it does in death following exposure to X-rays. Therefore, studies were undertaken to define the importance of infection as a contributory cause of death following exposure to neutrons, as well as to elaborate some of the mechanisms associated with the post-irradiation events.

The radiation source used for all of the studies described in this report was the Walter Reed Research Reactor (WRRR) which provided a 5.5 to 1 mid-body neutron-to-gamma ratio. For total doses under 1200 r the dose rates were 68 r/min from fast neutrons, 17 r/min from thermal neutrons and 15 r/min from gamma rays. For total doses above 1200 r all dose rates were greater by a factor of 10. Groups of ten mice were placed inside specially built, miniature "lexan" isolators and exposed in the reactor. Standard procedures were employed to preserve the respective bacteriologic states of the mice throughout the course of the experiments. A monorail system was used to move the mice to and from the exposure position - approximately sixteen inches from the core of the reactor - while the reactor was operating at a steady state.

Female ICR mice, 12-14 weeks of age, obtained from a commercial source were used for all studies.

1) Lethal effectiveness of neutrons in germfree animals. The first study was designed to measure the relative sensitivity of germfree and conventional animals to neutron irradiation. Groups of mice were exposed to neutron doses ranging from 275 rads to 500 rads and were observed for thirty days. Percent mortality as well as mean and median survival times were calculated for each dose and the median lethal dose values (LD-50/30) for both groups were computed. The LD-50/30 for germfree mice was 414 rads; for conventional mice - 328 rads. The difference between these values is statistically significant. The LD_0 to LD_{100} range for conventional animals lies between 300 and 400 rads; for germfree animals it lies between 400 and 500 rads. There is no overlap between the two. The relative resistance is 1.3 (the ratio of the respective LD_{50} values 414/328), which indicates that bacteria-free mice require thirty percent more neutron irradiation than the control animals to show the same gross biological effects. For any given dose, germfree animals survive two or three times longer than the control animals.

This relationship was demonstrable even at highly supralethal doses, after which survival was limited to a few minutes or hours (see paragraph 2 below). It is possible that the very rapid component of cellular recovery mechanism is greater in the germfree animals and that a factor in conventionalized animals (resulting from bacterial contamination) retards the operation of this rapid repair. This study model may be useful in elaborating the fundamental mechanisms which are responsible for dose-rate phenomena, a matter of great importance in clinical radiation therapy, and the effect on troops of exposure to ionizing radiation under combat conditions in nuclear warfare.

It is likely that several mechanisms are responsible for the differences in survival between germfree and conventional mice. Some mechanisms may predominate in very short lengths of time (at high doses), others at intermediate times and dose rates, and further ones over protracted lengths of time. Little is known about these relationships and further study is clearly indicated.

2) Responses of germfree animals after neutron irradiation. A second study attempted to determine: (a) whether the germfree state affects the response of animals exposed to supra-lethal doses of neutron radiation, (b) whether the response of germfree and conventional mice is similar if expressed by post-irradiation survival time as a function of neutron radiation dose, and (c) whether the effect of the germfree state on neutron dose-response is similar to the effect seen after exposure to X-ray.

Germfree and conventional mice were exposed to graded doses ranging from 500 rads to 50,000 rads. The time of death was recorded and both mean and median survival times were calculated. After each dose, germfree mice lived longer than conventional mice. For doses below 10,000 rads there was no overlap between the two groups; all irradiated conventional mice died before the first death occurred in germfree animals. Exposure to doses higher than 15,000 rads reduced the differences between the two groups although the average survival time of the germfree mice continued to be longer than that of the conventional mice. In addition to quantitative differences (i.e., survival time), qualitative differences were seen. Regardless of the radiation dose, symptoms of radiation damage such as diarrhea, depression, tremors, convulsions,or incapacitation were always seen in the conventional animals before they appeared in germfree animals.

3) Comparative pathology of irradiated germfree animals. The third investigation, a comparative study of the pathology of the small gut of germfree and conventional mice exposed to neutrons, was an effort to relate the radioresistance of germfree animals to the difference in the rate of development of radiation induced lesions. The uptake of tritiated thymidine was used as an index of cellular damage and the rate of migration of tagged cells was correlated with survival time and radiation damage. Two dose levels were chosen based on information gained in the first two studies. A dose of 375 rads was administered to half of the mice (this dose is fully lethal to conventional mice but is a high sub-lethal dose for germfree mice). The remaining mice were exposed to 1000 rads (this is a supra-lethal dose to both germfree and conventional mice although the germfree animals survive one and one half times longer than conventional mice). Groups of animals were sacrificed at four different time intervals during the first post-irradiation day and daily thereafter for the next two weeks. This project is currently underway and the data are not yet available. This study is being done in collaboration with the Division of Experimental Pathology.

4) Hematologic changes in irradiated germfree animals. A fourth study, currently underway, has been closely coordinated, with the study described in part 3) above. After both doses, and at each of the time intervals noted, blood samples were obtained to examine hematologic changes in both groups. These data are also not yet available.

Summary and Conclusions.

The use of electron spin resonance spectrometry to measure free

radicals seems likely to prove highly productive in several widely divergent areas.

a. Fundamental information has been obtained concerning the damaging effects of ionizing radiation. Similarities between molecular damages induced by irradiation, freezing,and exposure to oxygen at high partial pressures have been found.

b. Free radical measurements in teeth and fingernails appear to offer a reasonably reliable biological dosimeter.

c. Reproducible patterns of change in ESR spectra in liver tissue after death have been demonstrated and may offer a means to determine time of death in forensic medicine.

d. Measurement of ESR spectra in liver tissue after death is being evaluated as an indicator of liver viability in organ transplantation. A collaborative study with Dr. Starzl at the Colorado General Hospital is currently underway.

Experimental efforts to isolate and identify specific kinds of physiochemical lesions induced in vivo by ionizing radiation have continued. New information on the biological significance of radiation-induced breakage of chromosomal DNA has been obtained through study of DNA strand breakage in several strains of E. coli. The relationship between radiation lethality and single strand DNA breakage appears to be complex, and further studies of this relationship are likely to reveal fundamental information concerning cellular repair mechanisms, as well as biologically significant molecular injury resulting from ionizing radiation.

Studies in higher organisms have continued. The effect of radiation on the behavior of ants has been further studied, and confirms that irradiation produces strikingly similar alterations of combative behavior patterns in ants and primates, although much higher doses are tolerated in ants.

In mammals, a complex series of studies has been undertaken to examine the combined effects of radiation and traumatic injury. Whole body irradiation four days prior to wounding produces a marked delay in wound healing and increases mortality, while whole body irradiation four days after wounding reduces lethality of this combined injury. This finding has fundamental implications in the evaluation and treatment of a soldier who has been both irradiated and traumatized. Protection of bone marrow by shielding, transplantation of bone marrow,or treatment of the animals with aminothiols during irradiation all appear to induce a more normal wound healing pattern. These studies demonstrate the role of bone marrow in wound healing. Further studies will be designed to identify the type of marrow cells which produce this effect and explore the mechanisms which are involved.

Wound healing was similar in animals that were germfree, treated with

antibiotics, and conventional, but a lower mortality rate was observed in irradiated, wounded germfree animals which confirms the role that secondary infection plays in mortality after irradiation.

Studies of irradiation injury to the exteriorized small bowel of rats showed a patchy pattern of mucosal damage which is not seen after whole body irradiation or abdominal irradiation of the bowel in situ. Subsequent studies produced considerable evidence that this results from compromise of vascular perfusion and local anoxia produced during the surgical procedure. The anoxic areas of relatively undamaged mucosa appeared to be relatively hypertrophic, but efforts to demonstrate a humoral factor which stimulates gut regeneration have been inconclusive. Further studies are in progress.

The decrease in sensitivity of germfree mice to X-irradiation which was previously demonstrated here has now been confirmed after neutron irradiation. Germfree mice exposed to neutron irradiation survived longer than conventional animals, even at highly supra-lethal doses which were compatible with survival times of only a few minutes. These findings suggest that the germfree state may augment the capacity of cells to effect the very rapid phase of cellular repair after radiation injury. Further studies using this model may reveal significant information concerning repair mechanisms.

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