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US ARMY MEDICAL RESEARCH AND NUTRITION LABORATORY

Fitzsimons General Hospital

Denver, Colorado 80240

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U.S. ARMY MEDICAL RESEARCH AND NUTRITION LABORATORY Fitzsimons General Hospital Denver, Colorado 80240

US ARMY MEDICAL RESEARCH AND DEVELOPMENT TECHNICAL REPORT

1 July 1968 - 30 June 1969

The research conducted at the U. S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado was accomplished under the following projects:

5016 - 6.11, 01. A - In House Laboratory Independent Research

3A061101A91C - In-House Laboratory Independent Research

Work Units 049 - 057

5011 - 6.11.02. A - Defense Research Sciences, Army

3A061102B71P - Basic Research in Support of Military Medicine

> Ol - Biochemistry Work Units 058 - 062

3A061102B71R - Research in Biomedical Sciences

- 02 Internal Medicine Work Unit 055 Work Units 057 and 058 Work Units 061 - 064
- 05 Environmental Medicine Work Units 080 - 083 Work Unit 085 Work Unit 090

5028 - 6.21.10. A - Biomedical Investigations

ii

Projects (Cont'd)

3A062110A822 - Military Internal Medicine

Work Units 065 - 068 Work Units 071 - 074 Work Units 076 - 081

3A062110A827 - Military Environmental Medicine

Work Units 070 - 073

3A062110A830 - Bio-Sensor Systems

Work Unit 061

US ARMY MEDICAL RESEARCH AND DEVELOPMENT TECHNICAL REPORT

I July 1968 - 30 June 1969

SUMMARY

In-House Laboratory Independent Research - This program is instituted as one aspect of a broad approach to provide individual Army scientists and engineers an additional opportunity to maintain and increase their competence by doing original work in areas suiting their talents, thereby promoting a vigorous internal research program of the highest technical caliber.

Defense Research Sciences, Army - For research and development projects in the field of physical sciences to include general physics, nuclear physics, chemistry and mathematical sciences; engineering sciences to include electronics and energy conversion; life sciences to include biological and medical sciences and behavioral and social sciences.

Basic Research in Support of Military Medicins - This project includes basic biological research in the fields of biochemistry, biophysics, immunology, microbiology, pathology, pharmacology, physiology, radiobiology and zoology. The project supports selected research work which indirectly promises to assist the rest of the mission oriented AMEDD research program, both basic and applied.

Nochemistry: The objective of this task is to gain fundamental knowledge of the biochemical pathways involved in anabolism, catabolism and energy transformation, in order to understand and, perhaps influence nutritional requirements, antibody formation, action of chemotherapeutic agents, and metabolic responses to environmental stress, injury and special military hazards.

<u>Research in Biomedical Sciences</u> - The objective of this project is to obtain information by the techniques of clinical and basic research on injuries and diseases, except communicable diseases, commonly seen in soldiers, especially in field operations and overseas. The work is divided according to the major medical specialties.

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Internal medicine: The objective is to study, by basic research techniques in the laboratory, those diseases of soldiers in the field which are the special province of internal medicine in order to indicate possible approaches to improvements in treatment and prevention. These diseases include diarrhea, hepatitis, anemia, and altered metabolic states in which matrition plays an etiological or contributory role.

Environmental medicine: The objectives are to obtain basic information on the physiological responses of men and animals to climatic changes, especially to heat, cold and high terrestrial altitude, upon which may be based improved procedures for acclimatization, protection and treatment of injury resulting from exposure of soldiers to climatic extremes,

<u>Bio-Medical Investigations</u>, <u>Military Internal Medicine</u> - The objective is to improve existing methods of treatment of disease of military importance, such as acute or chronic viral and bacterial respiratory diseases, gastro-intestinal infections and abnormalities of gastro-intestinal absorption on feactions; to improve the management of certain military metabolic problems, such as post-traumatic nutritional deficiencies; and to study the soldiers' miritional status and adequacy of his diet.

Bio-Medical Investigations, Military Environmental Medicine - The objective is to develop better methods for the provention and treatment of diseases produced by the extremes of climate to which a soldier may be exposed.

<u>Bio-Sensor Systems</u> - The objective of this program is to provide the Army with an improved detector dog capable of tracking, detecting ambush, tunnels, weapons, mines and booby traps--and locating casualties in combat operations. Through a coordinated program of selective breeding, evaluation and veterinary research, a superior dog will be developed which has the specific desirable physical, behavioral and sensory characteristics, free of transmissible genetic defects, is responsive to military training, imposes a minimum logistical burden and can perform effectively on or off-leash in all climatic and operational environments. Work at USAMRNL has centered on the influence of the climatic environment on the working detector dog.

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ABSTRACT

ICT NO.3A061101A91CIn-House Laboratory Independent
Research.WORK UNIT NO.049The Mechanism of Body Temperature
Control by Adrenal Steroids.STUDY NO. 1To investigate the mechanisms
of body temperature control
particularly with regard to
adrenal steroids and the control
of exothermic reactions in
liver.

A patient with persistent fever has been investigated and it was found that his persistently elevated temperature could be controlled by means of dexamethasone and various uronic acids. It appeared that those uronic acids that were beta-glucuronidase inhibitors would decrease his temperature. From these studies it was hypothesized that there exists a regulatory mechanism in the liver whereby body temperature is controlled. This mechanism involved the ratio between conjugated and free etiocholanolone, with the ratio determined by the activity of beta-glucuronidase. The beta-glucuronidase in turn is controlled by the level of uronic acids which will inhibit the enzyme. If the enzyme is abnormal, e. g. resistant to inhibition by uronic acids, a greater proportion of total intrahepatic etiocholanolone will be in the free state. Free etiocholanolone produces fever. Dexamethasone in some way blocks the pyrogenic action of etiocholanolone.

There exist a number of patients with unusual or intractable fever often of a periodic nature and sometimes associated with disabling symptoms. It has been postulated that this periodic fever is due to some derangement of the mechanism involving etiocholanolone, beta-glucuronidase and the uronic acids. This possibility has been investigated in a number of patients with periodic fever whom we have had occasion to study. From the nature of the disease in the patients it can be concluded that periodic fever probably represents a heterogenous group of diseases which might be considered as different defects at different points along a common pathway. The reason for the periodicity is unknown. Why some patients have disabling symptoms and others are relatively asymptomatic despite the presence of fever is unknown. The variability in therapeutic response of individual patients to uronic acids and dexamethasone is also unknown. Nevertheless, we have uncovered a number of characteristics concerning this syndrome.

First, it is necessary to make very careful, frequent temperature measurements in order to detect fever which usually occurs in all of these patients most every afternoon and evening even to a small degree. Second,

The Mechanism of Body Temperature Control by Adremal Steroids (Cont'd)

with low grades of fever most of the patients are asymptomatic while symptoms occur when the degree of fever rises to higher levels. Thirdly, some of the patients respond to various uronic acids while others remain completely resistant. Fourth, most of the patients respond to dexamethasone though an occasional individual is quite resistant even to this. Fifth, there exist other categories of patients who appear to have periodic fever but who become hyperthermic with epinephrine infusions. For this we use the term stress or reactive fever. Sixth, there seem to be some individuals who get fever post-exercise. These last two entities may constitute a different category which requires elucidation.

BODY OF REPORT

WORK UNIT NO. 049

STUDY NO. 1

The Mechanism of Body Temperature Control by Adrenal Steroids.

To investigate the mechanisms of body temperature control particularly with regard to adrenal steroids and the control of exothermic reactions in liver.

PROBLEM :

The mechanisms for regulation of body temperature are not fully known. It is recognized that there exist temperature controlling centers in the hypothalamus which regulate the retention and loss of body heat. In addition, it is known that there are exothermic reactions occurring in the body which produce the heat. We are particularly interested in these exothermic reactions and believe there exist mechanisms for controlling the rate of these reactions thereby controlling the rate of heat production. Obviously, fever can occur if too much heat is produced, too little heat is lost or if both mechanisms occur. (This is, of course, an oversimplification, but because of space limitations it is not pertinent to go into detail). We have studied one patient with a persistent fever of 101.8° who had no diurnal variation. The patient was asymptomatic. His fever could be decreased by the administration of dexamethasone or various uronic acids which are themselves bets-glucuronidase inhibitors or lead to the formation of beta-glucuronidase inhibitors.

From these studies we have postulated that there exists a mechanism in human liver whereby the ratio of conjugated to free-etiocholanolone is regulated. Etiocholanolone is a normal adrenal steroid which has pyrogenic action when injected in the free state into normal humans. Betaglucuronidase is able to deconjugate the conjugated etiocholanolone to form free etiocholanolone. Therefore we postulate that the ratio of conjugated to free-etiocholanolone will determine whether or not a febrile state will occur. If there is too much free etiocholanolone then a febrile state will occur whereas if most of the etiocholenolone is in the conjugated form then the individual will be afebrile. How etiocholanolone produces fever is unknown. A defect in the mechanism of betaglucuronidase action, that is, deficiency of a normal inhibitor or the failure of the beta-glucuronidase to respond to an inhibitor could result in the formation of large amounts of intrahepatic free etiocholan. olone. Thus fever could result from either an overproduction of etiocholanolone by the adrenal, a defective conjugation of etiocholanolone, a failure of the beta-glucuronidase to be inhibited or the failure of the formation of an inhibitor. In addition, fever might occur if the mechanisms whereby etiocholanolone acts are extremely sensitive to what would be normal levels of intrahepatic etiocholanolone.

The Mechanism of Body Tomperature Control by Adrenal Steroids (Cont'd)

Periodic fever is a puzzling disease. The fever may be intractable, and may or may not be associated with various symptoms. By the study of these patients it might be possible to determine the nature of these so-called "periodic fevers", to categorize them into different entities and to discover the pathogenesis of the disease so that a rational therapy could be deviced and an understanding of body temperature control can be gained.

RESULTS AND DISCUSSION OF THE RESULTS:

A variety of patients with periodic fever have been studied. We have found that there are numerous differences between patients. One patient with fever but without symptoms developed fever with an epinephrine infusion. Epinephrine is calorigenic and increases oxygen consumption but does not ordinarily lead to the production of fever in normal humans. In this one patient, however, the infusion of epinephrine did cause significant fever and seemed to be related to a heightened sensitivity that this patient had towards epinephrine. This type of periodic fever then would be a so-called "stress" or "reactive fever." That is, there are a number of individuals who respond to environmental stress with epinephrine secretion to which they are inordinately sensitive with a heightened calorigenic response. Another patient with fever but with no symptoms was found to have fever after exercise. On investigation, we found that most normal individuals do not develop significant fever post-exercise and that a maximal amount of physical exertion is required to cause post-exercise fever. The mechanism of fever occurring after exercise is not clear. The muscle tissue may continue to have exothermic reactions after the exercise in excess of the rate of heat dissipation or the blood flow to the skin may decrease too quickly to permit adequate heat loss with a consequent increase in body temperature. This type of response shifts the emphasis away from etiocholanolone in the liver and directs it toward muscle tiasus.

In the study of these patients with periodic fever it has become evident that careful measurement of temperature every two hours is necessary in order to characterize the temperature pattern of the individual. It has been found that individuals with periodic fever have fever almost every day in the evening, sometimes only for a few hours, so that if careful measurements were not made, this fever would not be detected. This is essential in proving the diagnosis. Often these patients are asymptomatic and unsware that they have a mild fever. Symptoms usually occur when the fever becomes more prolonged or occurs at higher levels. There are occasional individuals, however, who have symptoms even when running a very mild fever.

Uronic acid has helped in one patient who has been followed for a period of over two years. Another patient failed to respond to glucuronic acid

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The Mechanism of Body Temperature Control by Adrenal Steroids (Cont'd)

The state where it is and the state of the state of the

or dexamethasone but does respond to glucaro, $(1\rightarrow 4)$ lactone which is a very potent beta-glucuronidase inhibitor. We currently have three patients with periodic fever under study. One is the patient who responds nicely to glucurunic acid who has been followed for over two years. The second is a young man who has had persistent recurrent fever without symptoms. The third patient is a lady with severe abdominal pain, multiple surgical procedures and persistent intractable fever. Her most recent febrile episode occurred in October 1968 and has been unrelenting since. Exploratory surgery revealed no cause for the abdominal pain whatever, such as scar tissue, adhesions, an internal hernia, etc. Fever has been persistent. She has been treated for tuberculosis since caseating granulomas were found in the lymph nodes draining the ileocecal region at surgery. However, this was thought to be arrested tuberculosis and not the cause of her abdominal pain and fever. Nevertheless, she was treated empirically with no change whatever in her clinical state. Glucuronic acid has been of no help in this patient. Interestingly, dexamethasone caused improvement in the temperature pattern, however the abdominal pain became worse.

Other studies are being carried out to characterize the beta-glucuronidase of liver which splits etiocholanolone glucosiduronide. It appears that the serum beta-glucuronidase activity has no effect on etiocholanolone glucosiduronide but the liver beta-glucuronidase is active in splitting etiocholanolone glucosiduronide. Serum glucuronidase levels do not appear to reflect the hydrolysis of etiocholanolone glucosiduronide by the liver.

We are also investigating the mechanism of action of etiocholanolone and comparing it to the action of piromen (Pseudomonas polysaccharide) which is another fever-producing agent. We have been investigating patients with hypopituitarism and have found that patients without pituitary function do not develop fever after injected etiocholanolone.

CONCLUSIONS:

We must conclude that periodic fever is a symptom of a heterogenous group of diseases. We currently have hypothesized that they result from different defects along a common pathway. It is necessary to measure temperature quite frequently in order to prove fever in some patients who have mild febrile states. Symptoms generally occur in the more severe febrile states and less often when fever is milder. There are, however, occasional patients with mild fever with maximum symptoms. We have elucidated an entity called "reactive" or "stress" fever which seems to be related to sensitivity to epinephrine. There also appears to be a condition in which fever occurs post-exercise. Some of our patients respond well to glucuronic acid, others better to dexamethasone, while some respond to neither. One patient studied so far responds to glucaro (1-4) lactone. However, this material is difficult to obtain and wust be chemically prepared in our own laboratory. The Hachanism of Body Tamperature Control by Adronal Statuida (Cont'd)

RECORPORTIONS:

The study of body comparature control in mon has proven to be quite fruitful. We are beginning to sort out the different periodic fevers, Thus, this is a fruitful area for research that should be continued.

PUBLICATIONS :

R.M. Herman, E.L. Overholt and L. Negler. Familial life-long persistent fever of unknown origin responding to dexempthseens and usonic acids. Amer. J. Not. 46: 142, 1969.

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ABSTRAC

PROJECT NO. 3A061101A91C

WORK UNIT NO. 050

In-House Laboratory Independent Research

Clinical and Laboratory Examinations of Mature German Shepherd Dogs Fed Rice Base Diets

STUDY NO. 1 Formulate and Test a Balanced Rice Dict Without Animal Protein Supplementation

The purpose of this study was to document the clinical and laboratory findings in active mature German Shepherds fed rice diets and to formulate and test a balanced rice diet using mineral and vegetable nutrient supplements. The information gathered would pertain to the advisability of using rice base diets for the military dog and give an estimate of how long a military dog will work while fed a rice diet.

Three mature Shepherds were fed ground, moistened uncooked parboiled rice as an exclusive diet. One had 60 ml/day of soy sauce added for flavor, another 30 ml. All three dogs were clinically healthy and vigorous and gained weight over the first 9 months. At 14 months, the dog not receiving soy sauce died following rapid weight loss. During the period of weight loss this animal was mildly anemic and had moderate to marked elevations in creatining phosphate kinase and serum glutamic -pyruvic transaminase. Signs of inanition were evident at necropsy, and microscopic examination revealed degenerating thyroid goiter, degenerative changes in adrenal cortex, fatty liver and other changes of even less specificity.

The dog that received 30 ml/day of soy sauce with his rice dropped below his initial weight after 9 months. Over the next 13 months, this animal lost 5.5 kg and was euthanized 22.5 months after rice diet initiation. At terminus the dog was emcciated. There was bilateral keratitis thought to be associated with Vitamin A deficiency (low serum carotene and Vitamin A values) and brown coloration of the intestine associated with Vitamin E deficiency.

The dog that received 60 ml/day of soy sauce with his rice was 2.5 kg below his initial weight at the time of euthanasia, after 22 months of rice diet. At necropsy there was brown coloration of the intestine as found in the other dog.

This work unit is being terminated due to (1) difficulty in procurement of suitable mature German Shepherds for a controlled study and (2) changing priority since inception of this study.

Mature Shepherds can subsist on rice alone for several months under conditions of cage rest or mild exercise. When deficiency signs occur they appear more related to vitamins or trace minerals than to major components as protein or total calories.

BODY OF REPORT

WORK UNIT NO. 050

Clinical and Laboratory Examinations of Mature German Shepherd Dogs Fed Rice Base Diets

STUDY NO.

1

Formulate and Test a Balanced Rice Diet Without Animal Protein Supplementation

PROBLEM:

1. In Viet Nam nutritional problems with military dogs were originally ones of supply, but the improvement of supply channels largely solved these. There still is a potential supply problem in more isolated areas. When standard bagged or canned ration was not available, diets composed of rice and various supplements were fed. Results with these diets were inconclusive.

2. We were interested in developing and testing a practical rice diet without animal protein supplementation. The following points appeared relevant: (1) If supply problems should arise in a major rice-producing country where military dogs are located, how would the dogs fare on a rice base diet? (2) In what form should the rice be fed? (3) Would a rice-based diet be nutritionally practical? (4) What are the most practical and satisfactory vegetable supplements for such a diet for military dogs in Viet Nam? (5) The acceptability of a rice diet to dogs accustomed to other foods could be critical if these animals have to be worked immediately but do not begin promptly to eat sufficient quantities of the novel diet.

We had determined from preliminary feeding trials that mature German Shepherd dogs would be fed ground uncooked parboiled rice. Parboiled rice was selected since it is more plentiful and has better storage qualities than brown rice. Parboiled rice is more desirable because of its higher Vitamin B content. For simplicity, it would be more desirable to feed the rice uncooked and more importantly, uncooked rice has a higher nutrient parcentage than cooked rice.

To determine the form in which rice would be fed, uncooked parboiled rice was fed to two German Shepherds for a week and uncooked parboiled rice with soy sauce was fed at the same time to two other German Shepherds. The two dogs on the rice diet last 7 lbs. (total for both) in 7 days and the two dogs on the rice and soy sauce diet lost 4-1/4 lbs. (total) over the sume 7-day period.

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The dogs on the "rice alone" ration refused to eat it during the 7-day period. The average daily consumption for the dogs on the rice and say souce for the 7-day period was 431 gm each. The soy sauce was used as flavoring. We determined later that the refusal to eat the "rice alone" was due to lock of moisture. Dogs confronted with an undesirable or different faod will eat it eventually - that is, it may take 2 days or more, but this delay for a working military dog would be undesirable. In limiting feeding, soy sauce appears to enhance acceptability of a ration of parbailed rice.

After examining the feces of the two dogs that were eating the rice it was apparent that there was less than optimum digestibility. The rice grains were only slightly smaller than when they were ingested. Grinding the rice prior to feeding it resulted in increased assimilation.

Ground parboiled rice mixed with water or say sauce was then fed to three mature German Shepherds according to the following daily schedule:

	Rice, gm	Soy sauce/454 gm	Water, ml/454 gm rice
Dog A (No. 989)	771	0	120
Dog B (No. 987)	771	30	30
Dog C (No. 985)	861	60	60

The dags were fed twice daily and usually ate all that they were offered. Drinking water was available ad. 115.

RESULTS AND DISCUSSION OF RESULTS:

Dog A: This dog's weight performance is charted in Figure 1. It actually gained 4.3 kg initially, held to at least its starting weight for 10 months, lost over the following 4 months and died on 26 October 68. Increased serum levels of creatinine phosphate kinase (CPK) and glutamic-pyruvic transaminase were recorded during September and October, as was low packed erythrocyte volume (PCV), (Table 1). This animal's erythrocytes were riboflavin-deficient when analyzed in October 1968 (analysis by Mrs. Yaye Herman, Chemistry Div., USAMRNL).

At necropsy (26 October 68) the dog was very thin with marked loss of muscle mass and body fat. Absence of periorbital fat was expressed in deeply-sunken eyeballs. The hair coat was rough.

Parenchymatous organs were reduced in size and the liver was pale with a diffuse yellow tinge. There were siderotic nodules on the surface of the spleen, a common and apparently inconsequential change in old dogs attributed to local chronic passive congestion. The changes seen at necropsy were compatible with general inanition. Microscopic examination revealed:

1. Degenerating, colloid-poor goiter of thyroid gland; its epithelium had been hyperplastic and was now undergoing degeneration and fibrosis; the gland was not enlarged.

- 2. Disorganization, degeneration and early fibrosis of adrenal cortex.
- 3. Fatty metamorphosis, liver.
- 4. Lipid vacuoles, hepatocyte nuclei, liver.
- 5. Lipofuscin pigment, hepatocytes, liver.
- 6. Hemosiderosis, Kupffer cells, liver.

7. Ballooning hypertrophy and hyperplasia, pigment epithelium, retina, focal.

8. Atrophy, giant cell formation and lack of mature sperm, seminiferous tubule, testes. (This was an adult dog.)

9. Atrophy and fibrosis, prostate.

10. Siderotic nodules, spleen.

11. Atrophy, skeletal muscle.

Atrophy of the various organs, fatty metamorphosis and marked lipofuscinosis of liver are associated with inanition of any cause. Multiple endocrine involvement (adrenal, thyroid) suggests more widespread disorder in this system but further conclusions cannot be drawn from the limited biochemical data in this pilot survey. The reduced size of the goiter-containing thyroid was particularly interesting. Follis, in his text in reference to mice on Remington's standard low-iodine diet, says, "Surprisingly, the only component of the diet which appeared to influence the size of the thyroid gland was sodium chloride, whose omission greatly reduced

the size of goiters, though thyroid hyperplasia was present even in its absence. "* In this dog with the small hyperplastic goiter, the diet consisted only of rice and water. Parboiled rice contains no iodine and has o low salt content, 9 mg sodium per 100 grams of rice. The two surviving dogs had received soy sauce. Soy sauce is produced by steeping soy bean products in brine, the salt probably containing iodine. The soy sauce fed was not analyzed for salt or iodine content. The earlier death of the dog not receiving soy sauce may be related to greater iodine deficiency if not sodium chloride deficiency. The swelling of retinal pigment epithelial cells indicates that there was a detached retina, antemortem.

Dog B: This animal did not drop below his original weight until 9 months after the start of the feeding trial. Over the next 13 months, this animal lost 5.5 kg of body weight and was euthanized on 10 July 69, after 22.5 months of the rice diet. This animal's peak weight was 9.2 kg higher than the starting weight, reached after approximately 4 months on the rice diet.

At terminus the dog was emaciated. There was gross evidence of muscle atrophy. All body organs were normal in gross appearance with the exceptions of the small intestine and both eyes. There was brown coloration of the intestine which was interpreted as a response to Vitamin E deficiency, and bilateral keratitis thought to be associated with Vitamin A deficiency (low serum carotene and Vitamin A values, Table 1).

Dog C: This dog was euthanized on 11 July 69. At death he was 2.5 kg below his starting weight, after being fed this ration for approximately 22 months. This animal's peak weight gain was 6.1 kg after 6 months on this diet.

The necropsy findings were unremarkable with the exception of brown discoloration of the intestine wall, interpreted as a response to Vitamin E deficiency.

The feces of the three dogs were of normal consistency except that Dog B had diarrhea during its period of weight loss (Figure 1).

These three dogs were exercised uneventfully on an outdoor dog exercising device from 1 April 1968 to 30 June 1968, 45 minutes per day, 3 days per week. Throughout the test feeding period these animals were placed in outside runs 5 days per week and remained in the runs from 2 to 5 hours daily.

*Follis, Richard H., Deficiency Disease, Charles C. Thomas, pg. 78, 1968.

It appears that weight loss in relatively inactive mature German Shepherds on a ground parboiled rice diet is more related to deficiency of essential trace nutritional factors than to protein inadequacy, since deficiency of the latter would result in more rapid loss of condition and weight than we observed in these three animals. It is interesting to speculate how mature German Shepherds would fare on a ground parboiled rice regimen with vitamins and minerals supplemented. This work unit is being terminated due to (1) difficulty in procurement of suitable mature German Shepherds for a controlled study and (2) changing priority since inception of this study.

CONCLUSIONS:

1. It is possible to maintain body weight and apparent good health in mature relatively inactive German Shepherds for a number of months by feeding ground parboiled rice and water, or parboiled rice and soy sauce, both diets with water ad. lib.

2. As the clinical condition of the dogs deteriorates, deficiency of riboflavin and Vitamin A become apparent. Deficiencies of other nutrients were not specifically determined but may have been present as well, e.g. iodine.

PUBLICATIONS:

None. An MRNL Laboratory Report is in preparation.

TABLE 1: Clinicopathologic Valu	TABLE	1:	Clinicopathologic	Value
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	Dog A	Dog B	Dog C	Control Dog
CPK units				(normal 09 units)
16 Sep 68	80	10	10	
24 Sep 68	60	15	25	
SGPT units				(normal <25 units)
12 Sep 68	67	-	-	
24 Oct 68	73	-	-	
PCV (%)				(normal 45-50%)
12 Sep 68	32	35	45	
24 Oct 68	32	38	56	
B-Carotene (mcg/10	(Im 00			> 9.7
16 Apr 69	-	0	0	-
Vitamin A (mcg/10	0 ml)			> 147
16 Apr 69	-	25	40	-



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ABSTRACT

PROJECT NO. 3A061101A91C

In-House Laboratory Independent Research

WORK UNIT NO. 051

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The Evaluation of the Natriuretic Property of 16-Alpha-Hydroxyprogesterone in Human Subjects

The following investigation was conducted under this work unit:

STUDY NO. 1: The Evaluation of the Natriuretic Property of 16-Alpha-Hydroxyprogesterone

16-alpha-hydroxyprogesterone is produced by the normal human adrenal gland. Natriuretic properties have been attributed to this steroid compound. A constant intravenous infusion of 16-alpha-hydroxyprogesterone was administered for 6 days into one normal subject and for 10 days into two additional normal subjects. The infusion of the steroid was preceded by an eight-day equilibration period in which the subjects received a constant high sodium-normal polassium diet which was continued throughout the entire study. This period was followed by a control infusion period and finally the period of infusion of the steroid which was compared to the control.

The first subject receiving 16-alpha-hydroxyprogesterone for 6 days was on strict bed rest in the supine position during all infusion periods. The other two subjects were allowed to ambulate during the infusion periods.

In the first subject there was a kaliuresis associated with a tendency toward sodium retention resulting in an elevated urinary potassium to sodium ratio during the infusion of 16-alpha-hydroxyprogesterone. Urinary aldosterone excretion increased during the infusion of the steroid compared to the control period. These results were also found in subject two. The results were variable in subject three. It was felt that change in posture and renin-angiotensin activity there may have negated the effect achieved in the other subjects.

These preliminary data suggest that 16-alpha-hydroxyprogesterone opposes the action of aldosterone in normal subjects resulting in elevated levels of aldosterone when infused in face of a high sodium intake.

BODY OF REPORT

WORK UNIT NO. 051

The Evaluation of the Natriuretic Property of 16-<u>Alpha-Hydroxy-</u> progesterone in Human Subjects

PROBLEM:

16-alpha-hydroxyprogesterone is produced by the normal human adrenal gland. It has been reported by Janoski, et al., that this steroid is produced in large amounts in untreated patients with salt-losing congenital adrenal hyperplasia. Natriuretic properties have been attributed to 16-alpha-hydroxyprogesterone in various reports. The actual blood concentration necessary to produce a response in humans is unknown. The natriuretic effects of this steroid in humans had not been previously determined. 16-alphahydroxyprogesterone was prepared from 16-alpha-17-alphaepoxyprogesterone.

After constant electrolyte balance on a high sodium (250 mEq'day) normal potassium (50 mEq/day) intake was attained, three subjects received a control infusion. This was followed by a constant infusion of 30 mg'day of 16-alpha-hydroxyprogesterone for 6 days in Subject I and for 10 days in Subjects II and III. Subject I was restricted to the supine position during all infusion periods; whereas, Subjects II and III were allowed to ambulate. Frequent blood samples and 24-hour urine samples were collected to compare electrolyte changes and aldosterone excretion during control versus the steroid infusion periods.

RESULTS:

In Subjects I and II there was kaliuresis, and urinary sodium retention resulting in an elevated urinary potassium to sodium ratio during the infusion of 16-alpha-hydroxyprogesterone. These changes were associated with a rise in urinary excretion of aldesterone during the infusion of this steroid. In Subject III a true balance was not attained during the control period and the variable results suggested the interplay of the renin-angiotensin system secondary to postural changes.

The findings in Subjects I and II during the infusion of 16-<u>alpha</u>hydroxyprogesterone are consistent with an opposition of this steroid to the action of aldosterone. The Evaluation of the Natriuretic Property of 16-<u>Alpha-Hydroxy</u>progesterone in Human Subjects (Cont'd)

RECOMMENDATIONS:

Additional studies are necessary to verify these findings and to establish a dose-response. Normal subjects should be studied only in the supine position to negate postural effects on electrolyte balance and aldosterone. Adrenalectomized patients are needed to establish a log-dose response of 16-alpha-hydroxyprogesterone to mineralocorticoid without the intervention of the physiological readjustments in subjects with intact adrenal glands. This work unit shall be closed in July 1969 because the principal investigator is leaving the Laboratory and Service.

PUBLICATIONS:

1. Janoski, A.H., M. Roginsky, N.P. Christy and W.G. Kelly. Hyperproduction of 16-alpha-hydroxyprogesterone in salt-losing congenital adrenal hyperplasia. Abstract No. 59. The 51st Annual Meeting of the Endocrine Society, New York 1969 (June).

2. Janoski, A. H., M. Roginsky, N. P. Christy and W. G. Kelly. Metabolism of 16-Alpha-Hydroxy C₂₁, Steroids III. J. Clin. Endocrin. and Metab. (In Press)

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ABSTRACT

PROJECT NO.	3A061101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	052	Coronary Blood Flow Studies

The following investigations have been or will soon be initiated under this work unit:

STUDY NO. 1:	Effects of Nitroglycerin and Amyl Nitrate on Coronary Blood Flow in Intact Dogs
STUDY NO; 2:	Effects of Selective Coronary Arteriography on Myocardial Blood Flow in Man
STUDY NO. 3:	The Response of the Coronary Vasculature and Tissue Fluid Compartments to Decreased Oxygen Supply
STUDY NO. 4:	Reflex and Direct Effects of Selective Coronary Arteriography in the Dog
STUDY NO. 5:	Effects of Coronary Receptor Activation on Renal Function

This work unit was established to investigate the effects of pharmacologic agents and environmental stress on the coronary circulation. Several methods of measuring coronary blood flow will be utilized including: (1) myocardial clearance of Xenon-133, (2) electromagnetic flowmeters, (3) indicator-dilution curves using labelled and nonlabelled tracers. In the past year two studies on the effects of selective coronary injection of radiopaque contrast agents have been completed. One study in man has confirmed that myocardial blood flow does increase transiently following coronary arteriography. Another study in intact dogs has shown that the hemodynamic responses to coronary injections of radiopaque media are in part reflex-mediated through activation of coronary stretch receptors. The results of the latter study have been submitted for publication. Experiments designed to determine the effects of activation of the coronary stretch receptors on renal function are presently underway.

BODY OF REPORT

WORK UNIT 052

STUDY NO. 1

Coronary Blood Flow Studies

Effects of Nitroglycerin and Amyl Nitrate on Coronary Blood Flow in Intact Dogs

5.

PROBLEM:

Until recent years, information concerning the responses of the coronary circulation has been derived primarily from isolated heart or heart-lung preparations of experimental animals or from postmortem studies in man. Unfortunately, the data obtained from these experimental situations, excluded from normal metabolic support, thermal regulation and autonomic nervous system and respiratory influences are not satisfactory for predicting hemodynamic responses in the intact animal or in man. Recently, however, several methods have been developed which allow the measurements of coronary blood flow in the intact, closed-chest dog or in man. This permits the study of the coronary circulation within an anatomically and physiologically intact cardiovascular system.

RESULTS:

Due to various technical difficulties we have not been able to proceed with this study. Because the principal investigator is leaving the service in the immediate future, this study is being withdrawn.

STUDY NO. 2

Effects of Selective Coronary Arteriography on Myocardial Blood Flow in Man

PROBLEM:

The administration of small amounts of hypertonic, radiopaque agents directly into the coronary artery has been shown in both the open-chest and intact anesthetized dog to produce an increase in myocardial blood flow. Whether this phenomenon occurs in man, particularly in the presence of coronary artery disease is unknown. Selective coronary arteriography is being utilized with increasing frequency in most cardiac catheterization laboratories, thus it is important to obtain as much information as possible about the effects of this procedure.

RESULTS:
Coronary Blood Flow Studies (Cont'd)

In 4 of 5 patients with, and in 2 of 5 patients without demonstrable coronary artery disease myocardial blood flow (measured by the Xenon-133 myocardial washout method) increased 10 - 82% above control values within one minute of intracoronary injection of Renografin-60. One patient with, and two without coronary artery disease showed no change in myocardial flow and the remaining patient without coronary disease responded to the injection with a 17% reduction in flow. Those patients showing an increase in flow had lesser control values (range: 39 - 58 ml/min/100 g) than those who showed no change or a fall (range: 72-82 ml/min/100 g).

STUDY NO. 3 The Response of the Coronary Vasculature and Tissue Fluid Compartment to Decreased Oxygen Supply

This study has been moved to Work Unit No. 081.

STUDY NO. 4

Reflex and Direct Effects of Selective Coronary Arteriography in the Dog

PROBLEM:

Transient cardiac slowing and rarely cardiac arrest have been observed following selective coronary artery injection of radiopaque contrast agents. The cause of this slowing has not, to our knowledge, been delineated. After preliminary experiments in closed-chest dogs revealed this to be a reflex phenomenon, a more definitive study was designed and carried out.

RESULTS AND DISCUSSION OF RESULTS:

Hyperosmotic solutions of a variety of substances including polyvinylpyrrolidone, sodium diatrizoate, methylglucamine diatrizoate, NaCl, glucose, sucrose, and mannitol produced transient sinus bradycardia and fall in arterial blood pressure when injected directly into the coronary arteries of the intact anesthetized dog. More consistent and marked responses were observed when injections were made into the left as compared to the right coronary artery. The same changes occurred with acute coronary venous hypertension

Coronary Blood Flow Studies (Cont'd)

induced by coronary sinus obstruction with a balloon catheter. The responses were not produced by coronary injections of isosmotic saline or injections of the various substances into the aortic root. left ventricle or pulmonary artery nor were they abolished by preheating the solution to body temperature.

The drop in blood pressure occurred even when the bradycardia was prevented by pacing or atropine. Concomitant with the fall in blood pressure, femoral blood flow increased indicating the former response was due in part to peripheral vasodilatation. Maximal left ventricular contractility (LV dP'dt) decreased with the fall in blood pressure, even if the bradycardia was prevented. In most, but not all, of the experiments this reduction in maximal LV dP/dt could be nearly or completely abolished by beta adrenergic blockade. It is thus probable that a reflex-mediated decrease in myocardial contractility contributes to the fall in blood pressure. All responses were abolished by bilateral vagotomy with the exception of the fall in pressure and maximal LV dP/dt produced by the radiopaque media or 5% NaCl. These agents exert a direct myocardial depressant action. Simultaneously determined indicator-dilution curves using ¹²⁵I-albumin and ¹³¹I-diatrizoate verified that the diatrizoate molecule leaves the circulation during its passage through the coronary vascular bed. Calculations derived from these curves demonstrated that injection of non-labelled diatrizoate produces a large increase in coronary blood flow. In patients undergoing selective coronary arteriography, the bradycardia, but not the fall in blood pressure occurring with coronary injections of radiopaque media, could be prevented by atropine. The commercial preparations of sodium diatrizoate (Hypaque 50%) usually produced more intense bradycardia than the preparation of methylglucamine diatrizoate (Renografin 60%).

It is concluded that the depressor effects of coronary injections of hyperosmotic solutions or coronary sinus obstruction are the result of activation of a reflex arc originating from stretch receptors in the coronary capillaries or small veins. The afferent limb of the reflex is bilaterally represented in the cervical vagosympathetic trunks of the dog. The efferent effects characterized in these experiments were increased vagal firing to the sinus node, decreased activity of adrenergic fibers to peripheral vascular alpha receptors and probably diminished adrenergic firing to beta receptors in the ventricular myocardium.

STUDY NO. 5

Effects of Coronary Receptor Activation on Renal Function

Coronary Blood Flow Studies (Cont'd)

PROBLEM:

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To determine the role, if any, of coronary vascular stretch receptors in the regulation of renal function, i.e., are these receptors physiologically important as central volume receptors.

RESULTS:

Work to date has shown that a diuresis accompanies activation of the coronary receptors. Additional experiments are currently being conducted to determine the mechanism(s) involved.

RECOMMENDATIONS:

1. Studies dealing with the relationship between renal function and the cardiovascular system should continue. High altitude exposure, pulmonary artery pressure and renal function are interesting correlates that should be investigated.

2. Basic research in cardiac physiology should be continued.

PUBLICATIONS:

1. Carson, R. P., and R. Lazzara. Hemodynamic Responses Initiated by Coronary Stretch Receptors with Special Reference to Coronary Arteriography. American J. Cardiology (Submitted for publication).

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PROJECT NO.	3A061101A91C	In-House Laboratory Independent Research
WORK UNIT NO.	053	Studies in Nutritional Myopathies

The following investigations have been conducted under this work unit:

STUDY NO. 1 Effect of vitamin E deficiency on the fatty acid profiles of phospholipids isalated from rabbit muscle sarcolemma STUDY NO. 2 Effect of vitamin E deficiency on the calcium uptake and AT Pase activity of tabbit grana

Due to personnel shortages, no significant progress has been made during this fiscal year. Since qualified personnel are not anticipated to be available in the immediate future, further studies in this area have been discontinued.

WORK UNIT NO. 053

STUDY NO. 1

Studies in Nutritianal Myopathies

Effect of vitamin E deficiency on the fatty acid profiles of phospholipids isolated from rabbit muscle sarcolemma

PROBLEM:

The exact metabolic role of vitamin E in humans and laboratory animals is unknown. One hypothesis considers vitamin E as an antioxidant essential for the maintenance of normal cell function and integrity. This hypothesis is consistent with the creatinuria and increases in serum enzyme activity observed during vitamin E deficiency in several species. It seemed important therefore, to ascertain the extent that the fatty acids bound to the phospholipids in muscle membranes (sarcolemma) were altered by vitamin E deficiency.

STUDY NO. 2

Effect of vitamin E deficiency on the calcium uptake and AT Pase activity of rabbit muscle grana

PROBLEM:

Muscle sarcoplasmic reticulum is characterized by high calcium uptake or binding activity with a concomitant rapid AT Pase activity. Several investigators have shown that release of calcium fram the sarcoplasmic reticulum is essential for muscle contraction whereas uptake or binding of calcium occurs during muscle relaxation. Since vitamin E deficiency in the rabbit is characterized by a complete relaxation of the hind limbs, it seemed important to investigate the calcium uptake and AT Pase activity of several muscles in both deficient and control animals.

RESULTS AND CONCLUSIONS:

Due to departure of the principal investigatar and other personnel shortages, na significant progress has been made during this fiscal year. Since qualified personnel are not anticipated to be available in the immediate future, further studies in this area have been discontinued. Should personnel again become

Studies in Nutritional Myopathies (Contⁱd)

available, appropriate aspects of these studies would be considered for continued investigation under Work Unit No. 060: Basic Studies of Nutrition and Metabolism.

PUBLICATIONS: None

Several manuscripts based on findings reported in previous Annual Progress Reports are being prepared. Resulting publications will be subsequently indicated under Work Unit No. 060: Basic Studies of Nutrition and Metabolism.

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PROJECT NO. 3A061101A91C

WORK UNIT NO. 054

STUDY NO. 1

Animal Tissue A Sequential Study of the Ultracellular Changes Within the Hepatic and Reticulo-Endothelial Cells Following Administration of Parenteral Lipid Emulsions

In-House Laboratory Independent Research

Ultrastructure of

To study sequential cellular alterations during uptake, accumulatian and assimilation of lipid globules after intravenous fat administration. Reticuloendothelial and hepatic cells are of primary concern and the development of "intravenous fat pigment" and pigment granulomas are of particular interest.

Intravenous fat emulsion (10% cottonseed oil) was administered to 105 rabbits at the rate of 20 cc/kg body weight. Administration of the single full dose requires approximately 90 minutes. Sequential sacrifice began 5 minutes after initiation of administration and continued at intervals up to 8 weeks. Animals were sacrificed and tissues from liver, spleen, lungs and kidneys were collected and prepared for examination by light and electron microscopy. The time relationships from uptake of lipid to establishment of pigment and granulomas will be determined.

Collection of specimens from 105 rabbits has resulted in 2,520 epon-embedded specimens for electron microscopic examination and representative tissues for light microscopy as well. The electron microscopic examination of these specimens has been delayed due to personnel shortages and studies of higher priority.

WORK UNIT NO. 054

STUDY NO. 1

Ultrastructure of Animal Tissue

A Sequential Study of the Ultracellular Changes Within the Hepatic and Reticulo-Endothelial Cells Following Administration of Parenteral Lipid Emulsions

PROBLEM:

Fat emulsions could be a very useful component of parenteral alimentation regimens. Emulsions evaluated to date produce a pigment in reticulo-endothelial cells and hepatic parenchymal cells. The functional significance of this pigment has not been established.

Previous electron microscopic studies have shown that alterations exist at both the cell surface and within the cell, in association with uptake and assimilation of parenterally-administered fat emulsions. These previous studies have been sporadic with respect to total dose and time after administration.

A sequential examination, beginning during infusion and extending for some period after a standardized dose, has not been reported. This study will follow rabbits, three per time interval, from 5 minutes after initiation of a single 90-minute administration to 8 weeks, following the presence of lipid globules in capillaries to their entrance into the cell, their assimilation and the possible development of pigment and granulomas.

The tissues have been collected, epon-embedded and filed. Further processing of these tissues has been delayed due to personnel shortages and studies of higher priority.

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PROJECT NO.	3A061101A91C	In-House Independent Laboratory Research
WORK UNIT NO.	055	Development of a Semantic Auditory Conditioning Apparatus

The purpose of this investigation was to develop a reliable method for producing vasomotor, galvanic and myographic reflexes to conditioned stimuli along bipolar dimensions of meaning. Different types of unconditioned stimuli and reflexes were to be used to overcome idiosyncratic responsivity of different subjects. Parameters of conditioned stimulusunconditioned stimulus (CS-UCS) interval, conditioned stimulus (CS) and unconditioned stimulus (UCS) intensities and durations and subject awareness were to be studied.

The study has been terminated due to the departure of both the principal and associate investigator.

WORK UNIT 055

 Development of a Semantic Auditory Conditioning Apparatus

PROBLEM:

The purpose of this investigation was to develop a reliable method for producing vasomotor, galvanic and myographic reflexes to conditioned stimuli (CS) along bipolar dimensions of meaning. Different types of unconditioned stimuli (UCS) and reflexes were to be used to overcome idiosyncratic responsivity of different subjects. Parameters of CS-UCS interval, CS and UCS intensities and durations and subject awareness were also to be studied.

RESULTS:

No formal results were obtained. Feasibility studies on a limited number of subjects gave promising results. The study has been terminated due to the departure of both investigators early in FY 69.

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PROJECT NO. 3A0611

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In-House Laboratory Independent Research

WORK UNIT NO. 055

tr. 6.

Metabolic Interrelationships

Three studies on dogs have been initiated to determine the mechanisms by which the adrenal glands respond to hypotension. Preliminary information is incomplete.

Further studies under this work unit were terminated due to the sudden transfer of the principal investigator.

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WORK UNIT NO.

055

PROBLEM:

Hemorrhage and drug-induced hypotension is associated with increased secretion of catecholamines. However, the mechanism by which the adrenal gland responds to the reduction in blood pressure is not fully determined. It is partly mediated via the central nervous system. An attempt was made to determine whether or not the adrenal gland responds directly to changes in blood pressure at the local level. Acute reduction in blood pressure to the adrenal glands can be achieved using a balloon (Dotter-Lucas), inflated above the renal arteries to create a gradient.

In the anesthetized dogs, reduction of blood pressure by approximately 50mm Hg produces a reduction in pulse, slight increase in plasma glucose concentration, and a marked increment in plasma free fatty acid concentration. The mechanism by which plasma free fatty acid concentration is increased during Arfonad (trimethaphan) hypotension was to be elucidated by using carbon-14 labeled free fatty acids in tracer amounts to determine turnover of plasma free fatty acids, by measurement of catecholamine secretion during the hypotension period, and by the use of propanolol, a beta blocking agent.

A study will measure the turnover of apoprotein of serum lipoproteins as influenced by plasma expanders. The turnover of the apoprotein will be determined using selenium -75. The latter isotope has been shown to be extremely useful when used in tracer amounts to determine turnover of proteins.

RESULTS AND DISCUSSION OF RESULTS:

Three experiments have been performed on dogs in an attempt to determine the mechanism by which the adrenal glands respond to a reduction in blood pressures. In addition to the analyses of catecholamines, plasma glucose and free fatty acids, measurements have been made on blood pressure, heart rate and cardiac output.

Metabolic Interrelationships (Con^tt)

CONCLUSIONS:

Three studies have been completed on dogs, dealing with the mechanisms of enhanced secretion of catecholamines during hypotension. The analytical procedures for determination of catecholamines, free fatty acids and cholesterol are now in operation.

While the proposed studies are deserving of further investigations, this work unit is being terminated due to the departure of the principal investigator.

RECOMMENDATIONS:

None.

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PROJECT NO. 3A061101A91C

In-House Laboratory Independent Research

WORK UNIT NO. 056

Behavioral Pharmacology and Psychophysiology

The following work is being accomplished under this work unit:

STUDY NO. 1: Aversive vs. Appetitive Conditioned Responses to Drugs in Rats

A technique for the evaluation of drug or nutritionally indiced body states is being developed for rats and guinea pigs. The first part of this study has been accomplished by studying the reactions of guinea pigs to meperidine hydrochloride (Demerol). The study on rats is in progress; after the completion of this half of the study, the data obtained from the rats and guinea pigs will be evaluated.

WORK UNIT NO. 056

STUDY NO. 1

Behavioral Pharmacology and Psychophysiology

Aversive vs. Appetitive Conditioned Responses to Drugs in Rats

PROBLEM:

There have been numerous attempts to condition drug effects to neutral stimuli. These attempts have met with very limited success. A new technique has been developed which appears to be highly successful in pairing a neutral stimulus to a drug effect. Further, it appears that the subjects¹ (Ss¹) reaction to the neutral stimulus after pairing can be used as an index of their aversion or attraction to the active drug. It is, therefore, the purpose of this series of studies to further develop this evaluative technique and apply it to a variety of nutritional and drug-induced states.

RESULTS AND DISCUSSION OF THE RESULTS:

There has not been an adequate amount of data analyzed to date to warrant a full report of results. There have been indications, however, that this evaluative technique is successful. More data are needed for a full discussion of these results.

CONCLUSIONS:

No conclusions can be reached at this point in the research,

RECOMMENDATIONS:

This series of studies should be continued until this technique can be either fully accepted or rejected as an evaluative tool. If it is accepted a specific protocol will be established to use the technique to study nutritional deficits in humans.

PUBLICATIONS:

None

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PROJECT NO. 3A061101A91C

In-House Laboratory Independent Research

WORK UNIT NO. 057

Parameters Associated with Learned Avoidance Responding

The following investigation is being conducted under this work unit:

STUDY NO. 1: Elects of Pre-Avoidance Shock, Overtraining, and Intersession Delay Upon the Frequency of an Active Avoidance Response During Reacquisition in Rats

Training an organism to avoid a noxious stimulus can be accomplished through the use of a laboratory procedure in which the performance of a motor response, made promptly after onset of a warning signal, prevents the occurrence of the noxious stimulus. The avoidance paradigm most frequently used consists of an initial phase for original training of the avoidance response, a second phase for control of forgetting, and a final phase for assessing reacquisition of the avoidance response.

Primarily, the project will attempt to investigate variables associated with learned avoidance behavior. Variables selected for study include: frequency of anticipatory motor response, amount of training and length of intersession delay between acquisition and reacquisition.

At present, an initial protocol for the work unit has been approved and all necessary equipment has been ordered. No results have yet been obtained.

WORK UNIT NO. 057

STUDY NO. 1

Parameters Associated with Learned Avoidance Responding

Effects of Pre-Avoidance Shock, Overtraining, and Intersession Delay Upon the Frequency of an Active Avoidance Response During Reacquisition in Rats

PROBLEM:

Numerous studies have attempted to assess the effect(s) that various avoidance-training variables have on avoidance retention. In general, evidence indicates the retention of an avoidance response is enhanced if the avoidance response is similar to a previously acquired response, if either few or many avoidance training trials are administered, and if the length of time between acquisition and subsequent reacquisition test of avoidance retention is short. Few, if any, attempts have been made to study the interaction effects of avoidance training variables already investigated. Of particular importance is the determination of the optimal parametric levels for the experimental variables associated with avoidance retention, as well as the determination of the interaction between number of avoidance training trials and the length of the intersession, acquisition-reacquisition delay period.

RESULTS AND DISCUSSION:

Pilot studies designed to establish the procedure for manipulating level of anticipatory-motor-responding (i.e., degree of similarity between the avoidance response and a previously acquired response) have been delayed due to lack of equipment.

CONCLUSIONS:

None

RECOMMENDATIONS:

Continue the work unit until determination of the optimal parametric levels for selected avoidance variables can be made.

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PROJECT NO.	3A061102871P	Basic Research in Support of Military Medicine
TASX NO.	01	Biochemistry
WORK UNIT NO.	058	Molecular Biochemistry

The following investigations have been conducted under this work unit:

STUDY NO. 1 Radiation effects in biologically active proteins.

STUDY NO. 2 Radiation effect in small organic molecules.

STUDY NO. 3 Procedural development for amino acid and peptide analyses.

- STUDY NO. 4 Procedural development for enzymic assays.
- STUDY NO. 5 Stabilization of protein structures towards destruction of biological activity.
- STUDY NO. 6 Relationships between protein conformation and biological activity.

STUDY NO. 7 Enzymic activities during thiamine nutrition states.

1. Aggregation has been shown to be a major radiation-induced change in ribonuclease, trypsin and lysozyme. Aggregates can be formed from active monomer molecules and activity is retained in the aggregates so formed from both lysozyme and trypsin in the presence of hydrogen sulfide gas.

2. New disulfide containing peptides have been isolated from an inactive insoluble lysozyme aggregate formed during irradiation showing that disulfide bond rearrangement does occur.

3. Analytical techniques have been perfected for measuring the products and extent of product formation from oxalic acid samples under the influence of ionizing radiation.

4. Semi-automated procedures have been developed to obtain satisfactory and reproducible peptide yields from hydrolysates of lysozyme containing large cystine peptides. Automated amino acid analyses at the level of 0.001 micromole are being perfected for use in characterizing small amounts of peptides.

Moleculor Blochemistry (Cont' d)

5. Sensitive manual procedures have been adapted and are in use in determining N- and C-termini in 0.001 micromole quantities of peptides. These procedures involve identification of dansyl amino acid derivatives from either dansylated peptides (N-terminus) or from carboxypeptidase-treated peptides (C-terminus).

6. Completely automated methods have been devised for lysozyme and four proteases which catalyzed hydrolysis of peptide bonds at pH 7.1. These methods made possible extensive studies of radiation stability in lysozyme and trypsin derivatives.

7. Ribonuclease, trypsin and lysozyme have each been cross-linked with several bifunctional reagents. Retention of native activity is good and, frequently, increased stability is noted towards heat or radiation. This technique is being explored in detail as the basis for a general radio-protective measure.

8. Proteolysis rates have been used to detect configurational changes occurring during radiation. A previously undetectable configurational change has been noted in a radiation product having an activity, amino acid composition and chromatographic behavior identical with that of native lysozyme.

9. Modifications have been proposed to improve the reliability of transketalase assays an an indicator of thiamine nutrition states.

WORK UNIT NO. 058

STUDY NO. 1

Molecular Biochemistry

Radiation Effects in Biologically Active Proteins

PROBLEM:

The immediate objectives of this study are to purify and chemically characterize radiation products from trypsin, ribonuclease, lysozyme and soybean trypsin inhibitor. The ultimate goals are to thus determine the modes of radiation inactivation in these and other enzyme proteins.

RESULTS AND DISCUSSION OF THE RESULTS:

Soluble and insoluble aggregates have been observed to be the principal inactive products formed upon irradiating crystalline lysozyme, trypsin and ribonuclease in vacuo. Soluble aggregates have been cleanly separated from active monomer materials by gel filtration on P-100 columns. This separation also permits quantitation of aggregate formation. Such products are formed in increased amounts as a function of radiation dose indicating that aggregates are true radiation products from these proteins.

The presence of H_2S during irradiation decreases aggregate yield from ribonuclease but at least doubles aggregate yield from lysozyme and trypsin. In the case of the latter two enzymes soluble aggregates produced in the presence of H_2S retain significant activity levels. These results indicate the free radical scavenger, H_2S , promotes aggregate formation from active monomers in which free radicals have been produced by irradiation.

Studies have continued on characterization of the insoluble inactive aggregate formed from lysozyme during irradiation. Previous studies in this Laboratory indicated the probability of disulfide bond rearrangement as a major mode of radiation damage. Accordingly, attention has been given to characterization of disulfide containing peptides from partial enzymic hydrolysates of the inactive insoluble lysozyme aggregate. An immediate difficulty was encountered in obtaining a reproducible set of hydrolysis peptides from the radiation products and from native lysozyme itself. This problem has been partially resolved by employing a prolonged digestion period of 2 days and a combination of pepsin and alpha-chymotrypsin as the proteolytic agents. The remaining variability appears to be due to two recognizable factors: (1) variations in the specificity and activity of available chymotrypsin preparations and (2) variations in chromatographic behavior of commercial phosphocellulose preparations used

Arclecular Biochemistry (Cont'd)

in large scale column separations of hydrolysate peptides. Both sources of variation are now minimized by purchasing large quantities of the affected materials.

Hydrolysate peptides have been resolved into single entities and groups of 2 to 5 peptides on preparative phosphorylate cellulose columns. The peptide fractions therefrom have been characterized in terms of size (Number of amino acids present), arginine content, disulfide content and amide content. At least two hydrolysate peptides from the inactive insoluble lysozyme aggregate contain disulfide bonds and are not found in hydrolysates of native lysozyme. This indicates the probability of at least two disulfide bonds having been rearranged from their positions in native lysozyme. However, there still remains the possibility of further disulfide bond rearrangements as well as other chemical changes which may be responsible for loss of enzymic activity.

CONCLUSIONS:

Aggregation is a major radiation change in ribonuclease, lysozyme and trypsin. The extent of aggregation is affected by the presence of a gaseous free radical scavenger, H_2S . Aggregates can be formed from active monomer molecules and these molecular units retain some activity in the aggregates formed from both lysozyme and trypsin.

New disulfide containing peptides have been isolated showing that disulfide bond rearrangement does occur.

RECOMMENDATIONS:

Current chromatographic techniques should be utilized in studies of possible disulfide bond rearrangement in radiation-produced aggregates from typsin and ribonuclease. Because of well-known differences in amino acid sequence and chain conformation these enzymes must be studied in terms of optimum proteolysis conditions to obtain reproducible high yields of constituent peptides. The role and mode of aggregatian in other proteins needs to be assessed. In particular, soybean trypsin inhibitor and sperm whale myoglobin will be studied. The latter protein is of interest because it contains no cystine residues and thus would not be expected to form aggregates by rearrangement of disulfide bridges.

PUBLICATIONS:

Stevens, C.O., Long, J.L., and Upjohn, D.R. Radiation produced aggregation in crystalline preparations of ribonuclease, lysozyme and trypsin. <u>Proc. Soc.</u> Exp. Biol. Med. (in press). Molecular Biochemistry (Cont'd)

STUDY NO. 2

Radiation Effects in Small Organic Malecules

PROBLEM:

The current objective in this area is to determine the extent and the products of radiation-induced decarboxylation in small model compounds such as oxalic acid, aspartic acid and glutamic acid. Results of these studies may be useful in predicting the importance of decarboxylation reactions during radiation inactivation of proteins.

RESULTS AND DISCUSSION OF THE RESULTS:

This is a collaborative study with a professor and one graduate student at the University of Denver. It has been in progress for only 6 months so that only preliminary results are available at this time.

Approximately 400 samples of oxolic acid and salts of oxolic acid have been irradiated in the cobolt-60 source. Gas chromatography has been found to be the most useful technique in separating and quantituting the radiation products from avalic acid samples. The analytical technique perfected requires about 2 hours of operator time per sample. Completion of sample analyses is expected to provide a basis for postulating mechanisms for radiation decarboxylation of axalic acid.

CONCLUSIONS:

Preliminary studies have been in progress & months on an investigation of radiation-induced decarboxylation of oxalic acid. Analytical techniques have been perfected.

RECOMMENDATIONS:

Continuation of present studies should be fruitful in the next fiscal year.

PUBLICATIONS:

None

Molecular Biochemistry Conthal

STUDY NO. 3

Procedura: Disvetadment for Amina Acid and Peptide Anolyses

PROBLEM:

The primary goals here are: (1), to devise more reproducible procedures for peptide separation and detection and (2) to devise more sensitive procedures for determining amino acid composition and amino acid end-groups in proteins and peptides. The small amounts of peptides available from radiation studies is frequently so minute that amino acid analyses would have to be conducted at the 10^{-8} mole level. Limited availability of analytical support in this area dictates procedural automation when possible.

RESULTS AND DISCUSSION OF THE RESULTS:

The automated peptide analyzer system produced by Technican has been modified to achieve separation of cystime containing peptides in satisfuctory yields from proteolytic hydrolysates of lysozyme. A major modification is the use of phasphorylated cellulose columns and elution of these columns with sodium acetate buffers at pht values no higher than 5.5. Large peptides, particularly, are not bound as rightly to such columns as they are to the polystyrene based ionexchange resins normally employed. Thus, a source of low peptide recovery has been removed. Limitations have been placed on the pht of the eluting baffers since disulfide bonds in mixtures of large cystime containing peptides have been observed to rearrange spontaneously at pht values above 7.6 to give rise to new artifactual sets of peptides. A margin of safety is allowed by not operating in the pht region 5.5 to 7.0.

The Technicon peptide system has been further modified by performing the automated ninhydrin color reaction on effluent fractions from columns rather than as a continuous flow measurement on the effluent stream. During alkaline hydrolysis in the analytical train ammonia is produced from most peptides containing amide groups. This gas production has been observed to affect adversely the correlation in time between the hydrolyzed and unhydrolyzed analytical streams as they are printed out on the recorder. This has probably lead to erroneous interpretations of dotted in the recorder. This has probably lead to brought under certain control by the fraction collection and analysis technique.

The automated peptide system as modified has permitted reliable detection and size estimation in hydrolysates containing large cystine peptides. The system has already been part to use in assessing radiation damage in lysazyme.

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A modified factorison proceeding for among and analyses we are seen to except and bot to ase in Retermining the among and and contrar of a number of crase-inner derivatives of aboractions, lass some, myson and anyled integer minimum. Forcall length ise associations of the trade of an appendice in any realized to any the more precise quantitation of the level U.D. micromorely of anime action. Contain T-quartions for abscrutially necessary to advant on form about a the appendiction and the exception and anyled in the appendice of any to improve reproduction in the exception and anyled in the appendice of an improve reproduction in the exception and anyled in the appendice of improve reproduction in the exception and anyled in the appendice of improve reproduction in the exception and anyled in the appendic colorimeter monitoring the exacted flow areas of 440 multice deem eliminates to improve flow characteristics in the exacted flow areas of the application and the an positive and hystoxyphistics. The exacted areas of the application and the evaluation of the amine active content of most anticipated protein and positive amplies. Further, this most dication permitts the continues applied and any amine active analyzes systems with the instrument components presently available for this application.

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NN-staminal amina actial have been determined in paptic-stymatyssin particles from native lysesyme. The procedue involves neartion of each popticle with along/I allohide to form the dang/I-popticle from which is liberated the th-staminal amino actid as the dang/I derivative by total actid hydrolysis. This derivative to been isolated and identifies. By thin layer alronatography from each of the pepticles on popticle groups in Sureal. Results have been assential to beth determining the purity of a poptice fraction and establiciting where the popticle anighted from in the summa actid sequence of notice lyssayme.

C-terminal amine actic identifications presently involve sligesting each peptide with carboxypeptidese for a limited time fallowed by reaction of the amine actic so liberated with stary " chilander. The stary! denivatives are then isolated and identified by thin layer charactersphy. A visual semi-quantitative evaluation of the identified spare activities the C-termines on C-termini amine actic. This proceedure has itself the greatly the assignment of peptides to their sequence in notive lysogyme and now makes possible similar evaluations of peptides from an inective insoluble appreade factor.

CONNELLUSIONIS:

A semii-automated proceedure as new modified gilves satisfactory and reproducible peptide yields from Hydralyoutes of llysozyme containing large systime peptides. Automated amine avoid analyses can new be pentamed on an atmo-micro scale

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L.D. micromatic of most paptions, a Januitive namual procedures have been solution and and an in use for determining the and C-termini in indicted poptide functions.

RECOMMENDATIONS:

Further refinement of the analytical procedure for omino acid, is possible. Sample wale can be reduced by mother factor of 10 by reducing column clameter and, accordingly, increasing buffer flow rate. The necessary modffications are in progress.

The system for periode detection and sizing need to be applied to other protein systems. Since each protein yields a different and unique set of periodes upon protectives each protein presents a new analytical problem. Alteration of the elution buffer system may suffice to resolve periode mixtures other than these arising from lysozyme.

Wikewise, it seems desirable to apply the methods and experience achieved for No- and C-termini identifications in lyspzyra paptide, to peptides isolated from Hydrolyspites of other proteins othered by radiations.

PUBLICATIONS:

None

STUDY INC. 4

Procedural Development for Enzymic Assoys 5

PROBLEM:

This work has been directed toward, the automation of existing manual procedures for enzymic scrivity assays in cases of enzyme, being subjected to radiation or soften inactivating stresses. Such automated assays would permit examination of radiation effects for a largen number of biologically active proteins and, perhaps more important, under a wider number of experimental conditions than are now possible.

RESULTS AND DISCUSSION OF THE RESULTS:

A completely automated procedure has been devised for the assay of tryptic activity. Case in is the substance employed. Tryptic catalyzed hydrolyzis

Molecula: Biochemistry (Cont'd-

of peptide bonds is followed by measuring the increase in color observed upon reaction with ninhydrin. Technicon modules were employed for sampling, mixing of reactants, development of ninhydrin color and monitoring and recording the ninhydrin color changes. It was necessary to employ a Lauda constant temperature bath to achieve the precise regulation of temperature ($37^{\circ}C. \pm 0.01^{\circ}C.$) desired during incubation of the enzyme sample with substrate. Current capability of the method is 15 samples per hour. Results are reproducible within 2% of a level of 1 µg/ml. notive trypsin. Without use of this method studies of the radiation stabilities of a number of trypsin derivatives would not have been possible. In several preliminary studies the method has been used with little or no alteration to measure the peptidase activities of alpha-chymotrypsin, pronase and subtilisin.

A completely automated method has also been devised for assaying lytic activity of lysozyme. The substrate used is not completely soluble being a suspension of lyophilized Micrococcus lysodeikticus cells. This necessitates maintenance of the substrate solution in an ice both and continuous stirring to assure constancy of substrate concentration. The decrease in turbidity of the cell suspension caused by lysozyme samples is monitored in a Technicon colorimeter-recorder combination at 570 npu. A Lauda constant temperature bath was required for the desired precision in temperature regulation during substrate digestion. Current method capability is 15 samples per hour. Results are reproducible within 3% at a level of 2 µg/ml native lysozyme. Again, this automated method made possible studies of the radiation stability curves for various crosslinked lysozyme derivatives.

Attempts are being made to automate ribonuclease assays. However, the accepted manual methods require a precipitation step to remove undigested nucleic acid after incubation with the enzyme sample. Replacement of this precipitation step with dialysis has not yet resulted in a method with the desired sensitivity or reproducibility.

CONCLUSIONS:

Automated assay methods have been devised for lysozyme and four proteases which catalyze peptide hydrolysis at pH 7.1. These methods have adequate sensitivity and reproducibility and have made possible extensive studies of radiation stability in lysozyme and trypsin derivatives.

RECOMMENDATIONS:

Automated assay methods are desired for two additional proteases, pepsin and pepcin. Also, an automated assay procedure for soybean trypsin inhibitor

Molecular Biochemistry (Cont'd)

would facilitate radiation work on this material. Such automated assays should be possible by relatively modest modification of the existing method for trypsin. A refinement of the automated lysozyme assay would be possible by using a soluble substrate such as glycol chitin. The method would then be on a more sound theoretical basis since the ly-is of this substrate by lysozyme more nearly follows classic Michaelis-Menten kinetics.

PUBLICATIONS:

Stevens, C.O. An automated procedure for the measurement of lysozyme activity. A manuscript in preparation.

STUDY NO. 5

Stabilization of Protein Structures Towards Destruction of Biological Activity

PROBLEM:

The principal aim is to develop a treatment of biologically active proteins to promote increased stability in these compounds towards radiation damage or other stresses. Emphasis here is being placed upon devising an approach that may be more generally applicable to the broad classes of enzymes that occur in cells rather than an approach specifically applicable to such things as enzymes containing an essential sulfydryl group.

KESULTS AND DISCUSSION OF THE RESULTS:

Since the existing disulfide bridges in most proteins are known to be dominant stablilzing factors, it seemed profitable to explore the possibility of introducing additional co-valent cross-links between amino acid residues in the native structure. If this can be done without adversely affecting enzymic activity than such links could be expected to confer increased stability towards possible modes of radiation damage such as chain fragmentation or disulfide bond interchange.

To this end we have chosen the direct approach of using bifunctional reagents which react with the epsilon-amino groups of lysyl side chains. Since the distance between the lysyl side chains in the protein molecule is a governing but unknown factor, it has been necessary to test at least several reagents having functional groups separated by vorying distances. Three reagents have been used extensively and are listed with pertinent descriptive data below:

Mo¹ecular Biochemistry (Cont¹ d)

Abbreviation	Name	Functional Groups	Distance Between Functional Groups in angst <i>r</i> oms
PSC	Phenol-2, 4-disulfonyl chloride	2 (-C-SO ₂ C1)	9.6
FDP	1,5–Difluoro–2,4– dinitrobenzene	2 (-C-F)	7.2
FNPS	p, p' –Difluoro–m, m' – dinitro–di pheny l–sulfone	2 (-C-F)	13.0

The distance between functional groupings has been estimated by construction of space-filling molecular models. FDP and FNPS are available from commercial sources but PSC must be synthesized and purified in this laboratory prior to use.

Cross-linked derivatives of lysozyme have been prepared with all 3 reagents. Products retain varying amounts of activity and need to be purified further since they are probably mixtures of molecules having cross-links at different places in the molecule. The best retention of native activity has been observed upon cross-linking lysozyme with PSC. The cross-links so introduced increase stability to heat but exert no effect on radiation stability. Cross-linking with FNPS does increase radiation stability significantly but cross-linking itself results in about a 60% loss of native activity.

Results obtained upon cross-linking ribonuclease have been more notable. Ninety per cent of native activity is retained during cross-linking with PSC or FDP and both types of cross-links result in increased stability towards radiation. Increased stability is more pronounced at high dose levels. Crosslinking with PSC also confers increased heat stability on ribonuclease. The use of FNPS as a cross-linking agent results in about 60% loss of native ribonuclease activity. However, the cross-linked derivatives lose activity at only about half the rate as does native ribonuclease when subjected to radiation.

Trypsin has been cross-linked with PSC and the product retains 55% of the native activity. This derivative requires a 40% larger radiation dose to achieve the same inactivation seen in native trypsin by a given dose. Cross-linking of trypsin has also been accomplished with FNPS. Again, retention of native activity after cross-linking is poor but what little activity remains is destroyed at about half the rate as in native trypsin as a function of radiation dose.
Molecular Biochemistry (Cont'd)

Saybean trypsin inhibitor has been cross-linked with all three reagents. A detailed study af radiation stability has not yet been performed. An adequate rapid assay far this protein is under development.

CONCLUSIONS:

It is clearly feasible to crass-link enzyme proteins and still retain enzymic activity. The activities of some of these cross-linked derivatives are markedly more stable tawards heat or ionizing radiation. This approach is, therefore, a promising technique for improving stability of enzyme proteins towards denaturing stresses such as radiation or heat.

RECOMMENDATIONS:

The cross-linked derivatives of the 4 native proteins already prepared are available for purification and characterization. In particular it would be most desirable to know at least how many cross-links have been introduced into purified derivatives. Amina acid analyses are in progress to answer this question. Hopefully the position of the cross-links can also be determined but this is a long term goal.

To prove the utility and the general application of this protective technique towards radiation damage, these studies should be extended to other enzyme prateins. Pepsin, papain and sperm whale myoglobin are good condidates. It is arricipated that such studies can be performed with the technical assistance of undergraduate and graduate students from the University of Denver in a cooperative program now being established.

Further attention needs to be given to the use of bifunctional cross-linking reagents other than the three under current study. These three each contain an aromatic ring. A reagent containing an aliphatic chain between the two functional groups might be more easily talerated by both the enzymes and as a drug in therapy – the ultimate potential application of these reagents.

PUBLICATIONS:

1. Stevens, C.O., and Long, J.L., "4,4'-Difluoro-3,3'-dinitrophenylsulfone as a cross-linking reagent far lysazyme", Proc. Soc. Exp. Bial. Med., in press.

2. Stevens, C.O., and Sauberlich, H.E., "Cross-Linking agents and stabilization of lysozyme structure towards radiation", Fed. Proc. 28, 899 (1969).

Molecular Biochemistry (Cont¹ d)

3. Stevens, C.O., Long, J.L., and Sauberlich, H.E., "Effects of peptide chain cross-linking on enzymic activity", Proceedings of the Southwestern Regional Meeting of the American Association for the Advancement of Science, Colorado Springs, Colorado, May, 1969 (Abstract).

STUDY NO. 6

Relationships between Protein Conformation and Biological Activity

PROBLEM:

This study is directed towards delineation of the 3-dimensional structure of proteins with particular attention to those structural aspects essential to biological activity. Ionizing radiation along with other denaturing agents are regarded here as experimental tools.

RESULTS AND DISCUSSION OF THE RESULTS:

Most proteins in the native state are attacked slowly and only to a very limited extent by proteolytic enzymes. If the disulfide bonds in these proteins are cleaved to give an extended chain configuration proteolysis occurs rapidly. Denaturing agents such as heat have intermediate effects on digestion rates. By studying proteolysis rates we have been able to make estimates of the fraction of peptide bonds exposed to the environment upon subjecting an enzyme molecule to a denaturing treatment.

The number of peptide bonds exposed to the environment and therefore accessible to cleavage by trypsin or pronase can be measured by following the resulting increase in amino groups reactive with ninhydrin. The formation of peptides soluble in trichloroacetic acid has also been followed during proteolysis studies. Alterations in conformation have been detectable in six radiation products formed from lysozyme. Among the six was one product having almost the same enzymic activity as native lysozyme and exhibiting chromatographic behavior identical with that of lysozyme. It has been observed that a change in the rate of peptide bond cleavage is not always accompanied by a change in the rate of proteolytic release of trichloroacetic acid soluble peptides. The use of trypsin as the protease most consistently reveals differences between proteins being compared. Molecular Biochemistry (Cont'd)

CONCLUSIONS:

Proteolysis rate studies have been useful in detecting configurational changes occurring during irradiation. A suspected but previously unproven configurational change has been detected in a radiation product formed from lysozyme. This product has the same activity, amino acid content and chromatographic behavior as native lysozyme.

RECOMMENDATION5:

Results of proteolysis rate studies need to be supplemented with measurements of side chain reactivities with reagents such as hydroxynitrobenzyl bromide for rryptophyl side chains and tetranitromethane for tyrosyl side chains. As peptide bonds are exposed to the environment these side chains inay also be exposed but probably at a different rate. Exposure of tryptopl /i side chains to the environment usually results in disruption of the active sire in lysozyme. Differences in rates of proteolysis and rates of exposure of tryp - phyl side chains as a function of heat or radiation dose could be used to calculate the extent of unfolding permissible without loss of activity.

It will also be of interest to assess conformational changes in cross-linked enzyme preparations being synthesized in another study. Such preparations have been found to be more stable than native forms towards denaturing agents. If conformational changes in cross-linked derivatives also occur to a lesser degree than in native enzyme such information would provide a clearer insight into the degree of dependence of activity on conformational structure.

PUBLICATIONS:

Stevens, C.O., and Sauberlich, H.E., "Proteolytic digestion rates for products from gamma-irradiated enzymes", submitted to <u>Radiation Research</u> for publication.

STUDY NO. 7

Enzymic Activities During Thiamine Nutrition States

PROBLEM:

The aim here is to devise and evaluate an assay procedure which measures erythrocyte transketolase activity alone. The presently used assay procedure actually measures the net action of three enzymes: pentose isomerase, pentose Molecular Biochemistry (Cont'd)

epimerase and transketolase. The unproven assumption is made that transketolase is the rate-limiting reaction.

RESULTS AND DISCUSSION OF THE RESULTS:

The addition of substrate amounts of xylulose-5-phosphate to the transketolase assay system would be the most direct solution to the problem as stated above. However, the cost of this material has been determined to be prohibitive particularly for widespread clinical use of the procedure.

An extensive literature search reveals that it is time-consuming but feasible to prepare a concentrated solution of pentose isomerase and pentose epimerase. This preparation can then be added to the incubation mixture to be certain that non-rate-limiting quantities of these two enzymes are present during the assay incubation period. This second approach is probably the only practical solution to the problem at present.

CONCLUSIONS:

Concentrated pentose isomerase and pentose epimerase preparations can be used to improve the reliability of transketolase assays as an indicator of thiamine nutrition states.

RECOMMENDATIONS:

The proposed modifications of the presently used transketolase procedure should be tested experimentally as technical assistance permits.

PUBLICATIONS:

None.

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25 (11)Progresse: a)Normal subjects fed high carbohydrate diets have hypertrigiv-										
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maintained on a high (ructose diet developed a persistent hypertrigly eridemia, b)										
We are close to synthesizing certain bile steroid intermediates necessary for enzyme										
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Thus, we will study jeju al metabolism in hypoglycemia.										
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ABSTRACT

PROJECT NO.	3A061102B711P	Basic Research in Support of Military Medicine
TASK NO.	01	Biochemistry
WORK UNIT NO.	059	Basic Studies in Lipids

The following investigations have been conducted under this work unit:

- STUDY NO. 1: Fo determine the effect of varying carbohydrate diets on serum lipids in normal nien.
- STUDY NO. 2: To determine the effect of varying carbohydrate diets on serum lipids in rats.
- STUDY NO. 3: To determine the mechanisms whereby disorders of carbohydrate-induced hypertriglyceridemia occur.
- STUDY NO. 4: To determine the factors which regulate blood glucose in normal individuals and patients with abnormalities of blood glucose regulation.
- STUDY NO. 5: To prepare bile steroid intermediates by chemical synthesis.

Abstract. Human liver was obtained from patients undergoing cholecystectomy and the capacity to metabolize fructose and glucose was determined. Informed consent was obtained from each of the patients from whom liver was obtained. The capacity of human liver for the glycolysis of fructose was greater than that for glucose. Fructose was metabolized to fatty acids, carbon dioxide and glyceride-glycerol at a greater rate than glucose. The conversion of glucose to fatty acids was dependent on the medium glucose concentration. These results are similar to those previously obtained for the rat.

In normal men fed sucrose serum triglycerides increased. There is a variable pattern of response in that some individuals increase serum triglycerides early whereas, in others, the peak of triglycerides occurs later. If body weight is kept constant hypertriglyceridemia occurs. In one individual where body weight declined hypertriglyceridemia did not occur with a high sucrose diet. Clofibrate treatment during dietary-induced hypertriglyceridemia generally had no effect.

The administration of clofibrate, 2 grams per day, to normal males decreased the activity of jepunal glucokinase, glucose-8-phosphate dehydrogenase, fructokinase and fructose-1-phosphate and fructose-1, 6-diphosphate addiases. This effect is specific since 6-phosphoglucome acid dehydrogenase and hexokinase activities were not altered. In rats fed clofibrate the following enzymes were decreased: fructose-1, 6-diphosphate addiase, glucose-6-phosphate dehydrogenase, and acetyl coenzyme & carboxylase. NADP-malate dehydrogenase was significantly increased in the clofibrate-fed rat. In animals fed clofibrate, whether they were on a fructose or a glucose diet, there was a significant decrease in hepatic glycogen. Ratioisotopes were used to demonstrate the decreased conversion of glucose and fructose into glycogen.

Potassium therapy given to six obese patients undergoing a twoweek fast led to a significant improvement in glucose tolerance. This improvement was associated with earlier and greater plasma insulin responses. Fasting for two weeks with no polassium produced no significant change in glucose tolerance. Fasting with porassium supplementation maintained normal polassium balance and produced a slight rise in total body potassium. When patients were fasted without potassium a statistically significant negative potassium balance developed and the total body potassium fell significantly. It appears that potassium depletion during fasting plays a significant role in the deterioration of glucose tolerance. In healthy non-obese normal mailes, fasting for 48 hours produced an abnormal glucose tolerance test which progressed in severity as the fast was prolonged to 72 and 96 hours. After fasting resting plasma insulin values fell and the response during the glucose tolerance test was delayed. During a six day period of refeeding after fasting the glucose tolerance test still differed significantly from control tests at one day. At two, three and four days the glucose tolerance test was normal but became abnormall on days 5 and 6. When eight normal subjects were led a 500 callorie diet containing 85 grams of carbohydrate for three days no significant alteration of the glucose tolerance test developed.

In order to study bile southesis it is necessary to prepare bill steroid intermediates. For a long period of time we have been attempting to prepare some of the bile steroid intermediates leading from cholesterol to cholic acid. We have now devised a procedure which is not yet perfected, but which is partly successful in providing some of the bille steroid intermediates which can be used to prepare other

Bussie Studies in Liquids (cont d)

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intermediates. This will give a range of substances which can be used for the study of the biospathesis of the various bile steroids. The method still requires perfection of isolation of the various intermediates in the chemical synthesis so that pure substances can be used for each particular step.

BODY OF REPORT

work unit no. 059

ETLOS NO. LA

Basic Studies in Lipids

To determine the effect of warying carbohydrate diets on scrum lipids in normalmen.

PROBLEM:

It has previously been determined that the hypertrigly condemna that occurs with distary carbobydrate in rate and man and patients with endogenous hypertrighy ceridenia (so-called carbohydrate-induced hyperimply actudence) depends on the type and amount of dictary carbohydrate. Rescaloric anounts of fructose or success may increase plasma triglycavide concentration to levels greater than with nonfructose containing sugars. Studies in rat liver indicated that one possible reason for the difference between the effect of glucose and fructose on plasma trighteeride concentration was that fructose was a better precursor of hepatic fatty acids. The difference in the conversion of fructose and glucose to faity acids was related to the fact that the rate of hepatic glycolysis of fructose was preater than that for glucose. By increasing the rate at which glucose could be metabolized one could increase the rate of conversion of glucose to datty acids. Thus a differential conversion of sugars to bepatic fatty acids could be one of the mechanisms for the observed differential effects of different sugars on plasma trigly coride concentration in man. In order to examine this possibility, studies were done measuring the rate of incorporation of ¹⁴C₆-fructose and ¹⁴C₆-glucose into fatty acids, glyceride-glycerol and CO2, and the activities of hepatic enzymes were measured in human liver.

RESULTS AND DISCUSSIONS OF THE RESULTS:

Human liver was obtained from patients undergoing elective cholecystectomy after informed consent was obtained. The liver tissue was then assayed for various fructose and glucose metabolizing enzymes. It was found that phosphorylation of tructose by fructokinase was more than 5-fold greater than the total activity for glucose phosphorylation by glucokinase and hexokinase. Fructokinase, measured in six human livers had an activity of 27, 4 + 3, 6 mgmoles of substrate

metabolized min. mg of protein while total phosphorylation by glucokinase and hexokinase amounted to 4, 89 mumoles of substrate metabolized min., mg of protein in the same six livers. The activities of phosphofructokinase, fructose-1.6-diphosphate aldolase, pyruvate kinase, fructose-1-phosphate aldolase, triokinase and NADH and NADPH alcohol dehydrogenases were measured. The rate limiting step appeared to be at glucokinase and hexokinase. If lipogenesis is related to glycolytic rate then the conversion of fructose to fatty acids should be greater than that for glucose, This was tested by incubating liver slices with radioisotopicallylabelled fructose and glucose. In each of four experiments fructose was converted to a greater degree than glucose to fatty acids, carbon dioxide and glyceride-glycerol. This increased conversion of iructose to fatty acids as compared to glucose could not be accounted for by breakdown of glycogen since fructose was metabolized to glycogen to a greater degree than glucose. By increasing the concentration of glucose in the medium of incubated human liver slices one could increase the content of fatty acids, carbon dioxide, and glycerideglycerol production. Thus in one experiment a concentration of glucose of 5 mmole led to the formation of 17 mumoles of glucose recovered as fatty acid/gram of liver/90 minutes of incubation. whereas a 10 mmole concentration gave rise to 30.4 and a 30 mmole glucose concentration gave rise to 57.6 mymoles of glucose converted to fatty acid/gram of liver/90 minutes of incubation. These results indicate that the control of fatty acid production from glucose is more closely regulated at the glucokinase and hexokinase steps. It must also be taken into consideration that phosphofructokinase is a potential controlling step, even though the data show that phosphofructokinase in vitro has the same activity as fructokinase. It is possible that, in vivo, because of the allosteric properties of phosphofroctokinase, the activity is much less than seen in the in vitro assay. These observed effects of glucose and fructose in human liver tissue are consistent with the predictions derived from a consideration of the differential conversion of fructose and glucose to fatty acids and the effect of glucose concentration on hepatic fatty acid synthesis.

CONCLUSIONS:

This study has shown that the differential conversion of glucose and fructose to fatty acids in human tissue is consistent with the idea that fructose is metabolized at a greater rate to lipogenic substrates

than is glucose. Glucose is controlled at the glucokinase, hexokinase and potentially at the phosphofructokinase steps. Whereas fructose by-passes these steps at a much greater rate and is converted to lipogenic substrates at a much greater rate of glucose because of the inherent capacity of the metabolic pathway. These results in human liver are similar to those found in the rat and are consistent with studies in which dietary sucrose increases serum triglyceride concentration to a greater rate in human volunteers than an isocaloric amount of glucose.

RECOMMENDATIONS:

These studies should be followed with an attempt to explain what is the ideal dietary-carbohydrate substrate for man. Whether there are individual variations in the handling of sucrose-containing foods such that adverse effects may occur in time in some individuals is not yet known. Further attempts should be made to explore the mechanisms controlling the formation of lipids from carbohydrate sources.

PUBLICATIONS:

1. Zakim, D., R.H. Herman and W.C. Gordon, Jr. The conversion of glucose and fructose to fatty acids in the human liver. Biochem. Med. 1969, in press.

STUDY NO. 1B

To determine the effect of varying carbohydrate diets on serum lipids in normal men.

PROBLEM:

The feeding of large amounts of carbohydrates to normal subjects lead to an increase in the plasma triglyceride concentration. In a large number of patients with hypertriglyceridemia there is a direct relationship between the plasma triglyceride concentration and the carbohydrate content of the diet. Clofibrate has been effective in reducing the plasma lipid concentration in patients with endogenous (or carbohydrate-induced) hypertriglyceridemia. Because of the important action of this drug it is necessary to know what action this drug has on the plasma lipid concentration in normal individuals with carbohydrate-induced hypertriglyceridemia. For this reason

normal male volunteers were given high sucrose diets and then treated with clofibrate to see the effect on the serum triglyceride levels.

RESULTS AND DISCUSSIONS OF THE RESULTS:

Four normal male subjects were placed on a 2800 calorie diet containing 40% of calories as sucrose, 17% as casein, and 43% as corn oil for three days. On the fourth day the content of sucrose was increased to 80% with a reciprocal decrease in the corn oil calories. Serum triglyceride was determined periodically. After six months the same diets were repeated but the individuals were given 2 grams of clofibrate for one week. In each of the four subjects there was a large increase in the serum triglyceride concentration when the 80% sucrose diet was fed. The administration of clofibrate for one week had no effect on serum triglyceride concentrations while the subjects ingested an ad libitum diet. When the diets were switched from ad libitum to a 40% sucrose diet there was no change in the serum triglyceride concentration. However with the 80% sucrose diet plus clofibrate therapy there was an increase in the serum triglyceride concentration equal to that seen with sucrose alone. In one subject the peak serum triglyceride concentration was greater with clofibrate than without clofibrate. When clofibrate was discontinued there was an increase in serum triglyceride concentrations in three of the subjects whereas the triglyceride concentrations fell slowly in the fourth subject.

CONCLUSIONS:

Present studies show that sucrose-induced hypertriglyceridemia in normal males is not prevented by the administration of clofibrate in a dosage which lowers the serum triglyceride concentration in patients with endogenous hypertriglyceridemia. The results do suggest that in some normal individuals clofibrate may diminish the triglyceridemic response to sucrose. However, at the beginning of the study when the individuals were eating an ad libitum diet, clofibrate did not lower serum triglyceride concentrations. Whatever the mechanism of clofibrate action is, it does not seem to be related to the mechanism whereby sucrose increases serum triglycerides in normal human males.

RECOMMENDATIONS:

Other hypotheses concerning the mechanism of clofibrate should be entertained. Preliminary data seems to suggest that clofibrate interferes with the recirculation of plasma triglycerides. It is premature,

at this time, to draw firm conclusions as to the mechanism of action of clofibrate.

PUBLICATIONS:

1. Zakim, D. and R. H. Herman. The effect of clofibrate on the serum triglyceride concentration in normal males fed high sucrose diets. J. Ather. Res. 1969, in press.

STUDY NO. IC

To determine the effect of varying carbohydrate diets on serum lipids in normal human men.

PROBLEM:

Because of the effect of clofibrate in lowering serum triglyceride levels in patients with hypertriglyceridemia it seemed to be of importance to determine the effect of clofibrate and various enzymes in humans. Since human liver is generally inaccessible, particularly in normal individuals, it was decided to study the effect of clofibrate on gastrointestinal enzymes since jejunal tissue is accessible for investigation. Therefore, in individuals given clofibrate to determine the effect on sucrose-induced hypertriglyceridemia, jejunal biopsies were obtained and various enzymes in jejunal tissue were measured.

RESULTS AND DISCUSSION OF THE RESULTS:

In normal males fed 80% sucrose and then given clofibrate it was found ihat glucose-6-phosphate dehydrogenase, but not 6-phosphogluconic acid dehydrogenase, was decreased by clofibrate in human jejunum. There was some decrease in jejunal glucokinase but no effect on jejunal hexokinase. Fructokinase, fructose-1-phosphate and fructose 1, 6-diphosphate aldolases were also decreased in activity.

CONCLUSIONS:

The differential response of enzymes to clofibrate suggests that there is a group of enzymes sensitive to clofibrate while another group is resistant. A fruitful approach to the mechanism of action of clofibrate would be in studying the effect of clofibrate on varying enzymes involved

in the regulation of serum lipid levels. These studies demonstrate an approach to the study of drug action in the human. Since jejunal tissue can be easily obtained it would be simple then to measure the effect of various kinds of drugs in man on jejunal enzymes.

RECOMMENDATIONS:

Further studies on jejunal enzymes and the effect of drugs should be undertaken.

PUBLICATIONS:

1. Zakim, D., R.H. Herman, N.S. Rosensweig and F. B. Stifel. Clofibrate-induced changes in the activity of human intestinal enzymes. Gastroenterology 56: 496, 1969.

STUDY NO. 2A

To determine the effect of varying carbohydrate diets on serum lipids in rats.

PROBLEM:

Studies were undertaken in the rat to study the effect of clofibrate on the glycolytic enzymes and lipogenic enzymes on a high carbohydrate diet.

RESULTS AND DISCUSSION OF THE RESULTS:

The effect of clofibrate on glycogen and various glycolytic and lipogenic enzymes was investigated in rats fed a 70% fructose diet. It was found that the following enzymes were significantly decreased in activity in the clofibrate fed animals: fructose-1, 6-diphosphate aldolase, glucose-6phosphate dehydrogenase and acetyl-CoA carboxylase. NADP-malate dehydrogenase was significantly increased in the clofibrate fed animals. No change occurred in hexokinase, 6-phosphogluconic acid dehydrogenase and citrate cleavage enzymes in the liver of clofibrate-fed animals. In clofibrate-treated animals fed fructose or glucose there was significantly less hepatic glycogen per 100 grams of liver as compared to those animals not fed clofibrate. Animals pair-fed fructose diets, with or without clofibrate, had no significant difference in the hepatic glycogen content. In rat liver slices fructose was converted to glycogen quite well in non-clofibrate fed animals and to a greater degree than was glucose. However, in fructose and clofibrate-fed animals both fructose

and glucose were converted very poorly to glycogen in liver slices. Similar results were found for glucose and glucose plus clofibrate-fed animals. That is, liver slices from the glucose fed animals converted fructose to glycogen to a greater degree than glucose whereas in liver slices from glucose and clofibrate-fed animals both fructose and glucose were converted very poorly to glycogen. The same was true whether the animals were pair-fed or not.

CONCLUSIONS:

This study in rats parallels that carried out in human jejunal tissue and demonstrates a specific effect of clofibrate on various enzymes in rat liver. Other enzymes are unaffected while the formation of glycogen from fructose and glucose in clofibrate-fed animals is inhibited.

RECOMMENDATIONS:

The mechanism of clofibrate inhibition of $\frac{1}{2}$ lycogen synthesis should be investigated further to see whether this will account for the mechanism of action of clofibrate.

PUBLICATIONS:

1. Zakim, D., R.S. Pardini and R.H. Herman. The effect of clofibrate (ethyl-chlorophenoxyisobutyrate) treatment on glycolytic and lipogenic enzymes and hepatic glycogen synthesis in the rat. Biochem. Pharmacol. 1969, accepted for publication.

2. Herman, R.H., D. Zakim and F. B. Stifel. Effect of diet on lipid metabolism in experimental animals and man. Fed. Proc. 1969, to be published.

STUDY NO. 2B

To determine the effect of varying carbohydrate diets on serum lipids in rats.

PROBLEM:

Studies done previously showed that feeding a diet containing a large amount of fructose or sucrose did not increase the alpha-glycerophosphate concentration in rat liver in comparison to glucose or rat chow feeding. Other workers indicated that alpha-glycerophosphate is a quantitatively important metabolic product of fructose metabolism.

There is data in the literature to suggest that fructose produces more glyceride-glycerol than does glucose. In our studies the animals were fed ad libitum whereas the other investigators administered fructose by injection. Thus, we have investigated the effect of acute fructose loads on the hepatic alpha-glycerophosphate concentration in the rat liver. The data was compared with that for glucose injection.

RESULTS AND DISCUSSION OF THE RESULTS:

The intravenous injection of fructose (200 or 400 mg) into anesthetized rats leads to a prompt increase in hepatic alpha-glycerophosphate concentration. This increase is transient since the alpha-glycerophosphate concentration cannot be due to lack of substrate for fructokinase since the fructose concentration in the plasma is sufficiently elevated. Changes in the hepatic pyruvate concentration after intravenous fructose were parallel to those for alpha-glycerophosphate. Glucose injection did not lead to an early increase in hepatic alpha-glycerophosphate concentration. However, twenty minutes after the intravenous glucose the hepatic alpha-glycerophosphate concentration was nearly two-fold greater than the control values.

CONCLUSIONS:

These data indicate that the effects of fructose on hepatic fatty acid metabolism cannot be related to sustained increases in hepatic alphaglycerophosphate concentration. These data also show that under certain conditions the concentration of hepatic alpha-glycerophosphate may be greater after glucose than after fructose administration.

RECOMMENDATIONS:

Investigation of the differential handling of glucose and fructose by man and rat should be continued.

PUBLICATIONS:

1. Zakim, D. and R. H. Herman. The effect of intravenous fructose and glucose on the hepatic alpha-glycerophosphate concentration in the rat. Biochem. Biophys. Acta 165: 374, 1968.

2. Zakim, D. and R. H. Herman. Regulation of fatty acid synthesis. Amer. J. Clin. Natr. 22: 200, 1969.

STUDY NO. 3

To determine the mechanisms whereby disorders of carbohydrate-induced hypertriglyceridemia occur.

PROBLEM:

Patients with endogenous (carbohydrate-induced) hypertriglyceridemia have a serious defect in the regulation of serum triglycerides when exposed to dietary carbohydrate. Identification of this defect should lead to more rational therapy of the hypertriglyceridemic state and prevention of the untoward sequela which may occur. It may provide understanding of the mechanisms whereby serum triglycerides are regulated and a determination of the necessity of maintaining serum triglycerides at a specified level in any given individual. Metabolic studies in patients with various types of hypertriglyceridemia have been undertaken.

RESULTS AND DISCUSSION OF THE RESULTS:

Patients with carbohydrate-induced or endogenous hypertriglyceridemia have been studied. The studies to date are incomplete but have been designed first to prove that the patient has carbohydrate-induced hypertriglyceridemia and second that the individual responds to ciofibrate therapy. The various parameters that have been studied in these patients include: glucose tolerance test with glucose and insulin levels, serum triglycerides on specific carbohydrate diets with and without clofibrate, serum lipoprotein patterns before and during clofibrate therapy, and level of jejunal glycolytic enzymes before and during clofibrate therapy. Later the ability to mobilize free fatty acids with appropriate stimuli before and during clofibrate therapy will be examined.

CONCLUSIONS:

At the present time data are being collected and analyzed, hence no conclusions are possible. A number of patients with endogenous hypertriglyceridemia who respond to clofibrate have been located and will be studied.

RECOMMENDATIONS:

When the final results of these studies have been tabulated and

analyzed the mechanism of action of clofilwrate may be apparent and a reasonable working hypothesis of the nature of the defect in hypertriglyceridemia may be derived.

PUBLICATIONS:

None.

STUDY NO. 4A

To determine the factors which regulate blood glucose in normal individuals and patients with abnormalities of blood glucose regulation. Trick Manuscreek in the South States and Southern

PROBLEM:

During the starvation of obese individuals it is known that the glucose tolerance test becomes abnormal. It is postulated that the negative potassium balance of starvation may be responsible for this abnormality; if so, supplementation with potassium during fasting may correct the abnormal glucose tolerance tests due to starvation. Accordingly, obese patients undergoing starvation for weight reduction were studied, with and without dietary potassium supplementation.

RESULTS AND DISCUSSION OF THE RESULTS:

Potassium supplementation during a two week fast was associated with a significant improvement in the glucose tolerance test in six obese patients. Plasma glucose values were statistically significantly lower at 0, 90 and 120 minutes when compared to control glucose tolerance tests performed at the beginning of the fast. This improvement was associated with earlier and greater plasma insulin responses than observed when no potassium was given. Fasting for two weeks without potassium produced no significant change in glucose tolerance. Potassium balance in fasting subjects receiving the potassium supplementation was normal. There was no correlation between plasma free fatty acid values and glucose tolerance.

CONCLUSIONS:

This study suggests that the abnormality of glucose tolerance in obese individuals undergoing starvation is related in part to a negative

potassium balance and that supplementation with potassium leads to a positive balance and an improvement in giveose tolerance.

RECOMMENDATIONS:

This association between potassium balance and gueose tolerance should be further studied. Potassium is necessary for protein synthesis and in particular for enzyme synthesis. Hence, the ability to handle a glucose load may be devendent upon the maintenance of glucose metabolizing enzymes which in part may require a positive potassium balance. This may be of importance in individuals undergoing starvation during weight reduction programs. Accordingly, turther studies are contemplated.

PUBLICATIONS:

Anderson, J.W., R.H. Hernan, K.L. Newcomer. Improvement in guicose tolerance of obese patients after fasting when given potassiun supplementation. Amer. J. Clin. Nutr. 1969, accepted for publication.

STUDY NO. 4B

To determine the factors which regulate blood glucose in normal individuals and patients with abnormalities of blood glucose regulation.

and the state

PROBLEM:

Glucose toverance becomes abnorn al in obese people being fasted and is corrected by potassium. Since fasting in normal individuals also gives rise to an abnormal glucose tolerance test, a study was conducted to determine the effect of fasting and refeeding on the glucose tolerance of normal individuals. In addition, the effect of small amounts of carbohydrate calories on the glucose tolerance test during an otherwise reduced caloric intake was investigated.

RESULTS AND DISCUSSION OF THE RESULTS:

The changes in the glucose tolerance test of healthy non-obese male volunteers was studied during periods of fasting, refeeding and while on a 500 calorie diet. Fasting for 48 hours produced an abnormal glucose

tolerance test which progressed in severity when the last was prolonged to 72 and 96 hours. After lasting with refeeding the glucose tolerance test still was abnormal at day 1. The glucose tolerance test became normal at days 2. 3 and 4 and did not differ from the control glucose tolerance test. On days 5 and 6 the glucose tolerance test became distinctly abnormal. These abnormal glucose tolerance test became distinctly abnormal. These abnormal glucose tolerance tests occur at a time when the subjects were losing weight and were in negative socium balance. When eight normal subjects were fed a 500 calorie distinction of the glucose tolerance test developed.

CONCLUSIONS AND DISCUSSION:

The abnormality of glucose tolerance which occurs during fasting and is prevented by feeding suggests that the glucose tolerance in part is regulated by enzyme levels in tissues which utilize glucose. It is necessary to maintain enzyme levels to metabolize alucose at the normal rate. Fasting may lead to a decrease in enzyme levels so that an acute giveose load cannot be handled during the fasting state to the same degree as during the ted state. The increasing intolerance to glucose occurring during lasting would then be correlated with a decrease in the enzyme levels in the body presumably in the liver. The abnormality of glucose tolerance occurring during the first day of releeding would reflect the time required for induction of enzymes by the dictary substrates. The reappearance of an abnormal glucose tolerance test during days 5 and 6 is unexplained at the present time. These concepts are lurther strengthened by the fact that glucose intolerance was prevented by feeding only 500 calories. These results are consistent with the data that shows that dictary substrates increase benatic enzymes in the rat and increase glycolytic enzymes in the human jejunal epithelial cell,

RECOMMENDATIONS:

These studies should be continued to further elucidate what specific enzymes are altered as a result of fasting and refeeding and the mechanisms whereby diet maintains tissue enzyme levels.

PUBLICATIONS:

Anderson, J.W. and R.H. Herman. The effect of fasting and refeeding on the glucose tolerance of normal men. Metabolism, submitted for publication.

STUDY NO. 5

To prepare bile steroid intermediates by chemical synthesis.

PROBLEM:

The synthesis of cholic and chenodeoxycholic acids from cholesterol in the liver and the regulation of such biosynthesis is being studied in this laboratory. The exact steps in the biosynthetic pathway are not completely known and the regulatory mechanisms are noorly known. The requirement for bile steroid production is unknown. It is known that bile salts are important for the emulsification of dictary substances in the gastrointestinal tract, but the exact function of bile acids is unknown. In order to study these problems it is necessary to have bile steroid intermediates available. In general these are difficult to obtain. We are attempting the chemical synthesis of bile steroid intermediates to provide material for studies of the biosynthetic pathways and the mechanisms regulating the pathway. Feeding studies will be conducted with these substances to evaluate the effect on the gastrointestinal tract.

RESULTS AND DISCUSSION OF THE RESULTS:

The following chemical steps are being followed in order to prepare various bile steroid intermediates:



CONCLUSIONS:

Utilization of this particular set of steps has resulted in the formation of some of the cholic aldehyde. The Reformatsky reaction was not as efficient as it should have been since the aldehyde was not completely separated from the alcohol. Nevertheless a small amount of the bile steroid intermediate, trihydroxycoprostanic acid, was prepared. It has been concluded that each particular intermediate should be purified before the next step is carried out, particularly before carrying out the Reformatsky reaction.

RECOMMENDATIONS:

This synthesis has proven to be particularly difficult; nevertheless, CPT Gilbert P. Anderson, Chemistry Division, has been carrying out these studies for the past year and a half and has made excellent progress. Although the chemical problem has not been completely solved, the basic problem itself is solved. Concerted effort to accomplish the synthesis of these difficult substances is necessary.

PUBLICATIONS:

None.

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25. (U) Progress (Jul 68 - Jun 69): 1. Actinomycin is being used to evaluate the role										
of Vitamin D in the transport of Ca and P across the small intestine into the blood. 2.										
Conditions for isolating active polysomes and cell sap ensymes for amino acid incorpor-										
ation studies have been investigated. In vitro incorporation was improved by altering										
components of the energy system, monovalent cation levels, incubation time, concen-										
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DPM/mg. ployribosomal protein in only 2 min. Polysome structure was altered dras-										
tically in this short period and rate of incorporation decreased sharply after 2-4 man.										
3. Zinc deficiency caused an alteration in 35 metabolism in the epiphyseal plate										
and primary spongiosa regions of chick bone. 4. Copper form synthetic lindgrenite (a										
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ABSTRACT

PROJECT NO.	3A061102B71P	Basic Research in Support of Military Medicine
TASK NO.	01	Biochemistry
WORK UNIT NO.	060	Basic Studies of Nutrition and Metabolism

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Assess the role of vitamin D in promoting the absorption of calcium across the wall of the rat's small intestine.
- STUDY NO. 2 Study on the effects of amino acid deficiency, hormones and other factors on rat liver polysomes and amino acid incorporation.

STUDY NO. 3 Studies on mineral metabolism and interactions.

1. Vitamin D can increase the transport of calcium across the intestinal wall of a D-deficient rat; the administration of actinomycin D (A_D) has been reported to inhibit this effect of vitamin D. From this it has been proposed that vitamin D acts by inducing the synthesis of a protein in the intestine, which acts to increase the transport of calcium. Since our D-deficient animals with normal blood calcium levels appeared to have an adequate absorption of calcium, it appeared worthwhile to investigate a possible relationship between intestinal absorptian of calcium, vitamin D and A_D .

Using the methods and experimental design reported in the Annual Progress Report for 1968, the results obtained showed no inhibition by AD of vitamin D's effect on calcium uptake by the intestine. Since this was contrary to the results reported in the literature, further experiments are required to check and compare the findings in our laboratory with those found in another laboratory. These experiments will cover 2 points:

a. A demonstration of the effect of AD on phospholipid synthesis while measuring its effect on calcium uptake in D-treated animals. A reduction in ³²P incorporation in phospholipids of the intestine would demonstrate that AD is behaving as an inhibitor. The simultaneous effect on calcium uptake would reveal whether it is likewise inhibiting the vitamin D effect.

b. Determine the effect of varying certain components of the in vitro incubation medium on the relation of AD to calcium uptake.

It is important to answer the question as to whether vitamin D acts by inducing the synthesis of an intestinal protein which promotes the transport of calcium. Such an answer would determine the direction of future work on the mechanism of calcium transport in the intestine, and the role of vitamin D in this transport. In anticipation of future work, a procedure has been tested designed to measure adenyl cyclase enzyme in mucosal cells of the small intestine.

2. Improved methods have been developed for isolation of an in vitro amino acid incorporating system (polyribosomes and cell sap) from rat liver. The superiority of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer as compared to tris-HCI was demonstrated. The pH optima for extraction of polyribosomes and cell sap enzymes were established as 7.6 and 6.4, respectively. Deoxycholate at a final concentration of 1.67% in post mitochondrial supernatant yielded more active polyribosomes than any of the Triton detergents tested. The optimal potassium concentration for extraction of both cell sap enzymes and polyribosomes was 75 niM. The optimal magnesium concentrations were 5 and 3.75 mM for polyribosomes and cell sap enzymes, respectively. Sulfhydryl protectants (GSH at 3 mM) in the extraction media markedly enhanced the amino-acid incorporating ability.

3. Improved methods are described for assaying in vitro amino acid incorporation by polyribosomes and cell sap isolated from rat liver. Gel filtration of cell sap with Sephadex G-10 yielded a more active preparation for protein synthesis than gel filtration with Sephadex G-25, Bio-Gel P-2, P-4, or P-6. The optimal pH of the assay was 7.3. Addition of GSH to the assay medium had no effect on amino acid incorporation. A ratio of 22.5 µg cell sap protein/µg polyribosomal protein saturated the in vitro assay system, but o higher ratio was recommended. In vitro incorporation of amino acids was linear for only two minutes. Although incorporation wes maximal at 41°C assays were routinely performed at 37°C to approximate physiological conditions. A final Mg²⁺ concentration of 5.0 to 5.25 mM was optimal whereas the optimal K⁺ concentration was near 75 mM. An ATP-regenerating system was recommended since high concentrations of AIP were deleterious. A mixture of 20 L-amino acids at 10 mM each was added routinely to satisfy the requirement for amino acids.

4. Weanling rats were used in experiments designed to study the metabolic availability of copper from a preformed copper-molybdenum (Cu-Mo) compound, subsequently identified as a synthetic form of lindgrenite. The various treatments used were: basal diet (Group A); basal plus 6 ppm Cu as the sulfate (Group B); basal plus 6 ppm Cu as the Cu-Mo compound (Group C); and basal plus 6 ppm Cu as the sulfate and 6 ppm Mo (equal to the Mo in Group C) as sodium molybdate (Group D). In the first experiment when the weanling rats were placed directly upon treatment, there were no significant differences in either growth rates or

hemoglobin concentrations among the various treatment groups. At the end of the six-week study, serum ceruloplasmin oxidase activity (CPA) was significantly reduced in the Group C rats compared with the Groups B and D rats which showed no significant difference in CPA. Liver and kidney Cu concentrations of Group C rats were significantly lower than those from Groups B and D rats, but significantly higher than those in Group A rats. In a second experiment, the weanling rats were depleted of their tissue copper stores for two weeks prior to being placed upon treatment. In this study, there were no significant differences in growth rates. Hemoglobin concentrations were reduced in all rats during the depletion phase. Following placement upon treatment, Group C rats repleted hemoglobin at a significantly reduced rate compared with Groups B and D rats, while Group A rats remained at a reduced hemoglobin level. Serum CPA, at the end of the six-week treatment phase, was significantly lower in Group C rats than in Groups B and D rats which were not significantly different. Liver and kidney Cu concentrations in this copper depleted study were not significantly different among any of the copper supplemented groups. These results suggest that copper in the form of the Cu-Mo compound, synthetic lindgrenite, is metabolically less available, particularly for cerulaplasmin oxidase activity, than is copper from the sulfate salt.

Further, it appears that this phenomenon is unique to the Cu-Mo compound since an equal amount of molybdenum (as sodium molybdate) does not elicit a similar response.

5. The effect of zinc status of the chick on sulfate-sulfur metabolism in the area of bone elongation was investigated. Two experiments were conducted in which 10 μ Ci of ${}^{35}SO_4^{\pm}$ were given either orally or i.p. to chicks fed either a zinc-deficient (14 ppm) or zinc-sufficient (90 ppm) soy protein-cerelose diet for 4 weeks. The zinc-sufficient chicks were divided into 2 groups: (1) ad libitum fed; (2) restricted fed so that their average weight gain was approximately that of the zinc-deficient chicks. At 6, 12, 24, 48, and 96 hours after isotope administration, the chicks were killed and the epiphyseal plate through the hypertrophic zone and the primary spongiosa of the tibias were removed and analyzed for ³⁵S content. In these 2 regions, the peak ³⁵S activity (dpm/mg fresh tissue) occurred approximately 12 hours after isotope administration by either method and was significantly less in the zinc-deficient chicks when compared to either zinc-sufficient group. The hexosamina level per mg of fresh epiphyseal plate was not altered by zinc deficiency. A significant increase in the hexosamine level per mg of fresh primary spongiosa was noted. These results suggest that the metabolism of SO_A , an important constituent of mucopolysaccharide in the area of bone elongation, has been altered.

BODY OF REPORT

WORK UNIT NO. 060

STUDY NO. 1

Basic Studies of Nutrition and Metabolism

Assess the role of vitamin D in pramoting the absorption of calcium across the wall of the rat's small intestine

PROBLEM:

A. By the use of actinomycin D (AD), it was shown that an interference with the synthesis of mRNA from DNA resulted in an inhibition of vitamin D insofar as its effect on calcium transport by the small intestine. From this it was concluded that vitamin D was responsible for the synthesis of a protein required for the transport of calcium across the intestine. It follows that a D-free animal should have little or no capacity for this calcium transport. The experience in our laboratory demonstrated that rachitic D-free animals, presumably deficient in this calcium-transporting protein, had normal blood levels of calcium (9-10 mg/100 ml) and therefore appeared to have adequate absorption of calcium. Furthermore, despite adequate levels of calcium in the blood of these D-free animals, the administration of physiological doses af vitamin D resulted in a significant increase in both blood calcium (10-11 mg/100 ml) and phosphorus. The questions which appeared were:

(1) If adequate calcium uptake by the intestine required a protein whose synthesis was controlled by vitamin D, how could we account for the adequate calcium transport in aur animals which were D-free, rachitic, and presumably deficient in this protein?

(2) What would be the effect of administering AD to rats which already had adequate calcium transport? Would those animals still respond to vitamin D after AD treatment?

B. In anticipation of future work, time has been devoted to the development of an assay pracedure for measuring adenyl cyclase activity in mucosal cells of the small intestine. This enzyme has been shown to be responsible for the synthesis of 3' - 5' AMP from ATP; the 3' -5' AMP has been implicated in the transport of ions across cell membranes. Thus, if calcium transport in the mucosal cells of the small intestine is increased in response to vitamin D, while adenyl cyclase activity is simultaneously enhanced, a relationship may be sought between vitamin D, calcium transport by the intestine and adenyl cyclase activity.

Methods for measuring adenyl cyclase activity in vitro require tests for ATP and 3'-5' AM⁹ in the μ M and nM range, while those methods for measuring the activity of this enzyme under conditions more closely resembling those found in vivo require tests for ATP, 3'-5' AMP, AMP and ADP in the same range of concentrations.

RESULTS AND DISCUSSION OF THE RESULTS:

A. Results of the experiments with A_D administered to animals on high calcium, low phosphorus diets (Ca-1.2%:P0.06%) and low calcium, high phosphorus diets (Ca - 0.02% : P - 0.3%) were reported in the Annual Progress Report for 1968. Briefly, the results of our experiments indicated that A_D did not inhibit the action of vitamin D on the intestinal transport of calcium. This was contrary to the results reported in the literature. In the current year, the work with A_D was extended to animals treated with vitamin D before A_D . In agreement with previously reported work, those animals treated with vitamin D before A_D showed no inhibition of vitamin D by the antibiotic. Finally, as a control group, animals were fed Purina Lab Chow for 21 days before being treated with vitamin D and/or A_D . In this experiment, where the animals were not deficient in vitamin D, there was no effect of vitamin D administration nor was there evidence of an effect of A_D .

Because of the difference in results obtained in our laboratory compared with another laboratory, following the use of A_D then vitamin D_r current and future work is designed to repeat an experiment with the antibiotic and the vitamin measuring the effect on calcium uptake by the intestine, while at the same time demonstrating that the antibiotic is either inhibiting RNA synthesis, protein synthesis or phospholipid synthesis. This procedure should demonstrate that the antibiotic is effectively inhibiting other metabolic processes while its effect on vitamin D is being determined. The experimental design includes a comparison between different media in which the intestinal tissue may be incubated to assess the effect of different components of the media on the action of the antibiotic in vitro. These questions must be investigated before a definitive statement may be made regarding an inhibition of vitamin D by A_D .

B. By the application of some methods already in the literature and the development of a thin layer chromatographic separation of ATP, ADP, AMP and 3' – 5' AMP, it is now feasible to proceed with an experimental design to determine whether vitamin D affects adenyl cyclase activity in the mucosal cells of the small intestine.

CONCLUSIONS AND RECOMMENDATIONS:

A. The hypothesis that vitamin D acts to increase intestinal absorption of calcium by controlling the synthesis of a protein is of sufficient importance to warrant investigation. Such a protein might be an enzyme with specific catalytic functions which might provide an opening into understanding the mechanism for calcium transport. Also, it is possible such a protein could be non-enzymic in nature, having no catalytic function, yet participating in the transport of calcium, as tor example, the calcium-binding protein of the chick intestine reported in the literature. On the other hand, if the hypothesis is incorrect one must look elsewhere for the vitamin D effect. Definitive studies should provide some insight as to areas for future investigations with regard to the mechanism of action of vitamin D and the calcium transport mechanism in the small intestine.

8. The experiments relating adenyl cyclase, vitamin D and intestinal transport of calcium will be undertaken when the AD experiments are completed.

PUBLICATIONS:

Ziporin, Z.Z., G.J. Isaac, C.G. Liddle, and P.F. Waring. Effect of Actinomycin D on vitamin D-mediated uptake of ⁴⁵Ca by intestinal slices of rachitic rats. Fed. Proc. 28, 759 (1969) (Abstract).

STUDY NO. 2

Study on the effects of amino acid deficiency, hormones and other factors on rat liver polysomes and amino acid incorporation.

PROBLEM:

Following the earlier glucostatic and lipostatic theories of food intake regulation, it was postulated that the central regulatory mechanism may be influenced by the pattern of plasma amino acids. As a result, considerable work in the area of dietary amino acid imbalances has followed as reviewed by Harper (In Mammalian Protein Metabolism, Munro and Allison Ed., Academic Press, Inc., N.Y., 1964, Vol 11, pg. 87). Harper postulates it is the low plasma concentration of the limiting dietary amino acid that is responsible for depressing appetite and thereby growth rate. Recently, Leung and Kogers (Life Sci. 8: 1, 1969) have confirmed the presence of a food regulatory mechanism in the brain area signaling the rat to decrease food intake in response to a low critical level in the plasma of the

growth limiting dietary amino acid. This would after the supply of amino acids for in vitro protein synthesis and since the quality and quantity of dietary protein influences the protein content of the liver, it follows that a change in protein synthesis will result not only in liver but perhaps in other tissues such as skeletal muscle and brain tissue.

The basic unit of protein synthesis is the polyribosome formed by ribosomes attached to strands of messenger RNA (m-RNA) which code for amino acid sequences in peptide chains that are synthesized by polyribosomes. Amino acid availabilit; may alter the rate of protein synthesis by influencing the speed of attachment and detachment of ribosomes on the m-RNA strand. In addition, the lack of one amino acid required for in vivo peptide synthesis will alter polyribosome stability as evidenced by Wunner et al. (Biochem, J. 101: 417, 1966) when he left out dietary tryptophan from an amino acid mixture Torce fed to rats.

While it is true that an amino acid imbalance induced by a diet devoid or severely deficient in a single amino acid will induce anorexia, a decreased growth rate and changes in polyribosome structure, Harpers' theory of imbalance based on the plasma concentration of the limiting dietary amino acid does not explain why an amino acid imbalance derived from an excess of a single amino acid will cause anorexia and a decreased growth rate. In that case, the imbalancing dietary amino acid is present in high concentrations in the plasma which may or may not affect the appetite regulatory center in a like manner as low concentrations of a limiting dietary amino acid. Moreover, it is not known whether polyribosome structure is altered with this type of imbalance.

In order to study the effects of imbalances on in vitro protein synthesis, a reliable reproducable system is required. Several reports dating back to Zamecrik and Keller (JBC, 209: 337, 1954) have described a variety of methods employed for the isolation of rat liver cell sap enzymes or "soluble factors" and poly-ribosomes. These methods varied to the point that it warranted an investigation to establish optimal conditions for the isolation of an in vitro system and determination of its protein synthetic capacity. Too often an artifact of isolation procedures may mask a treatment effect or induce changes in biological activity of a system that may be misinterpreted as a meaningful observation. We have devised a system to study in vitro protein synthesis in liver that will allow incoreporation rates of 80,000 to 100,000 disintegrations per minute (DPM) of U-¹⁴C-leucine of polyribosomal protein, more active than most systems utilized to date. Other investigators are now using our methods to elucidate the requirements for an active muscle polyribosomal system.

RESULTS AND DISCUSSION:

The main criteria for determining the superiority of one procedure over another was how the changes in methods affected the ability of the in vitro system to incorporate U-14C-leucine into polyribosomal protein and the corresponding changes that took place in polyribosomal profiles as determined by isokinetic sedimentation analysis.

Experimental details are as follows:

Male Holtzman rats (110-140 gm) were fasted overnight prior to decapitation, the livers removed and passed through a tissur press prior to homogenization with a glass hand homogenizer and tellon pestel. All isolation procedures were carried out at 4° C. The liver supernatants were spun at 30,000 X Gravity (G) for 5 minutes and polyribosomes were spun in a like manner after the detergent treatment. Finally cell sap was spun over a 0.5 M sucrose cushion while polytibosomal post-mitochondrial supernatant (PSM) was spun over a cushion of 2.0 and 0.5 M sucrose (321,000 X G for 1 hr. and 45 min.).

According to Good et al. HEPES (N-2-hydroxypiperazine-N'-2-ethanesulfonic acid) buffer is superior to TRIS buffer for use in many biological systems due to its lower pK_{d} (7.55 at 20° c) and small temperature coefficient (.013 pH units/ °c). When our system was isolated in the presence of HEPES buffer, 7.3 × 10⁴ DPM's of U-1⁴C-leucine were incorporated per mg. of polyribosomal protein compared to 5.9 × 10⁴ DPM's of U-1⁴C-leucine for the system isolated with TRIS buffer. The medium (SHKM₁) used to isolate cell sap contained 250 mM sucrose, 50 mM HEPES, 3.75 mM Mg⁺⁺, 3 mM glutathione (GSH) (added fresh each day), 50 mM KCL and finally 25 mM KOH which was required to adjust the pH to 7.6. Immediately before use, the pt. was lowered to 6.4 to isolate cell sap. To isolate polyribosomes, a second portion of liver was added to a med'um (SHKM₂) almost the same as SHKM₁, except that 5 mM MgCl₂ was used and the final pH remained at 7.6.

In order to free membrane-bound ribosomes, deoxycholaie (DOC) at a concentration of 1.67% (w/v) in PMS yielded a more active preparation than did Triton X-100, N-101, X-114, and X-120. Furthermore, RNA values were 80 to 90% of the protein values in DOC treated preparations but only 50 to 60% in the case of Triton preparations. Polyribosome profiles of the latter indicated large amounts of high molecular weight material for active protein synthesis in all preparations but resolution of the peaks indicating low molecular weight material was poor. Moreover, the incorporation of $L-(U-{}^{4}C)$ leucine was about half of that observed with DOC treated material.

Following ultracentrifugation over sucrose, cell sap was passed through a column of Sephadex G-10 (1.5 x 13 cm.) to remove cold amino acid. A comparison of cell sap passed through columns of Sephadex G-10, G-25, Bio-Ge¹ P₂, P₄, and P₆, revealed that 40% more incorporation was allowed with the G-10 treated cell sap.

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The K^{*} and Mg^{**} levels in both the extraction and assay media are critical. Many investigators have used 25-50 mM K^{*} in their extraction media, however, our data indicate that isolating the in vitro system in 75 mM K^{*} permitted almost 3 times the level of incorporation obtained with systems isolated in 25 mM K^{*}. This may not be too surprising since the intracellular K^{*} concentration has been reported to be around 75 mM. Of equal importance was the concentration of Mg^{**} in the isolation media for polyribosomes (5 mM), cell sop (3.75 mM), and the incubation media (5 mM). If Mg^{+*} was gradually increased up to 15 mM in the polyribosomal isolation medium, a progressive decrease in the percentage of high molecular weight material was noted accompanied by a decrease in incorporating ability.

Contrary to the procedures utilized by many investigators, a sulfhydryl reagent was employed in our isolation media. GSH (3 mM) allowed the isolation of a more active cell sap prep for the promotion of in vitro amino acid incorporation while polyribosomal activity was influenced very little. Subsequently, no requirement for sulfhydryl could be demonstrated in the in vitro incubation. The addition of GSH to isolation buffers is preferred since it affords early protection of cell sap enzyme active sites.

In order to assay the incorporating ability of different polyribosome preparations, cell sap enzymes must not be a limiting factor. In our system, 4.5 mg, (.2 ml) of cell sap protein was used with 0.1 mg. (0.1 ml) of polyribosomal protein, however, a ratio of 22.5 parts of cell sap to 1 part of polyribosomes was just adequate to maintain a linear rate of incorporation for 2 minutes at 37° C.

The incubation med a (0.7 ml/assay) itself contained optimal levels of K (75 mM), Mg⁺⁺ (5 mM), sodium creatine phosphate (20 mM), ATP (1 mM), GTP (0.1 mM), 0.9 mM GSH (from isolation procedures), an equivalar mixture of 19 L amino acids (.19 mM), HEPES buffer (50 mM), and L-(U-14C) leucine (.8 uc). Following the 2 minute incubation pariod, protein was precipitated with cold 10% trichloracetic acid (TCA), washed once in hot TCA (5%) and 3 times in cold TCA and the pellet dissolved in 0.5 ml of hyamine hydroxide. The mixture was taken up in Brays solution for determination of activity incorporated.

CONCLUSIONS:

1. Improved methods have been developed for isolation of an in vitro amino acid incorporating system (polyribosomes and cell sap) from rat liver. The superiority of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfanic acid) buffer as compared to tris-HCl was demonstrated. The pH optima for extraction of polyribosomes and cell sap enzymes were established as 7.6 and 6.4, respectively. Deoxycholate at a final concentration of 1.67% yielded more active polyribosomes than any of the Triton detergents tested. The optimal potassium concentration for extraction of both cell sap enzymes and polyribosomes was 75 mM. The optimal magnesium concentrations were 5 and 3.75 mM for polyribosomes and cell sap enzymes, respectively. Sulfhydryl protectants (GSH at 3 mM) in the extraction media markedly enhanced the amino-acid incorporating ability.

2. Improved methods are described for assaying in vitro amino acid incorporation by polyribosomes and cell sap isolated from rat liver. Gel filtration of cell sap with Sephadex G-10 yielded a more active preparation for protein synthesis than gel filtration with Sephadex G-25, Bio-Gel P-2, P-4, or P-6. The optimal pH of the assay was 7.3. Addition of GSH to the assay medium had no effect on amino acid incorporation. A ratio of 45 µg cell sap protein/1 µg polyribosomal protein was utilized in the in vitro assay system. In vitro incorporation of amino acids was linear for only two minutes. Although incorporation was maximal at 41°C, assays were routinely performed at 37°C to approximate physiological conditions. A final Mg²⁺ concentration of 5.0 to 5.25 mM was optimal whereas the optimal K⁺ concentration was near 75 mM. An ATP-regenerating system was recommended since high concentrations of ATP were deleterious. A mixture of 20 L-amino acids at 10 mM each was added routinely to satisfy the requirement for amino acids.

PUBLICATIONS:

1. Huston, R.L., L.E. Schrader, G.R. Honold, G.R. Beecher and H.E. Sauberlich. Protein Synthesis: Factors influencing the isolation of an in vitro polyribosomal system from rat livers. (Submitted for publication).

2. Schrader, L.E., R.L. Huston, W.K. Cooper and H.E. Sauberlich. Protein Synthesis: Factors influencing in vitro incorporation of amino acids by rat liver polyribosomes. (Submitted for publication).

3. Schrader, L.E., R.L. Huston, J.A. Tillotson. Influence of cations on in vitro protein synthesis by rat liver polyribosomes. Fed. Proc. Vol <u>28</u>, pg. 755, 1969 (Abstract).

4. Huston, R.L., L.E. Schrader, W.K. Cooper, and H.E. Sauberlich. Factors influencing the ability of a rat liver-polyribosomal system to incorporate amino acids. Fed. Proc. Vol 28, pg. 755, 1969, (Abstract).

STUDY NO. 3

Studies on mineral metabolism and interactions

a. Interaction between copper and molybdenum and its relationship to ceruloplasmic activity

PROBLEM:

The study of copper nutrition, metabolism, and function is complicated by numerous interactions involving copper and other metals. One such interaction is the reciprocal antagonism between copper and molybdenum; molybdenum can precipitate copper deficiency and copper can alleviate molybdenum to kicity. The mechanism of this copper-molybdenum interaction has not been clearly elucidated. One attractive hypothesis is that copper and molybdenum form some sort of complex which may render both metabolically inactive. The study to be reported was designed to determine the metabolic availability of dietary copper from a preformed copper-molybdenum compound.

RESULTS AND DISCUSSION OF THE RESULTS:

Weanling, male rats (Spraque-Dawley) were used in two experiments concerning the metabolic availability of copper from a copper-molybdenum (Cu-Mo) compound, subsequently identified as a synthetic form of lindgrenite. In the first study, 48 rats were randomly divided into four treatment groups. The basal diet for this experiment is shown in Table 1. The treatments added to the basal diet are presented in Table II. Group A served as a negative control; Group B was the positive control; Group C received the Cu-Mo compound; and Group D was the control for Group C. The supplements provided 6 ppm copper (Groups B, C, and D) and 6 ppm molybdenum (Groups C and D). Body weights were recorded weekly and hemoglobin concentrations were determined at the second and fifth weeks of the six-week study. At the end of the experiment, the rats were sacrificed; serum was collected for ceruloplasmin oxidase activity (CPA) assay; and livers, kidneys, and spleens were excised for copper determinations (by atomic absorption spectrophotometry).

There were no effects of the various treatments upon either growth rates or hemoglobin concentrations. The effects of treatment upon CPA and tissue copper levels are summarized in Table III. The rats receiving synthetic lindgrenite, Group C, showed a significant depression in CPA compared with the other groups receiving copper supplements (Groups B and D). The lack of a difference between Group B and Group D seems to suggest that the effect observed in Group C is a function of the lindgrenite compound and not an influence of molybdenum alone. Since the Group C rats showed greater CPA than the negative controls (Group A), it appears that at least a portion of the lindgrenite copper is available for ceruloplasmin synthesis, even though it is not as available as is copper from the sulfate salt. Liver and kidney copper concentrations were significantly lower in the Group C rats than in the copper sulfate supplemented rats, either without or with sodium molybdate (Groups B and D, respectively). This observation might suggest that a part of the normal tissue stores of copper had been used for metabolic purposes. The same argument can be used for the lack of difference in tissue copper between Group B and Group D as was used for the non-significant difference in CPA of the same groups.

Since it has been reported that the copper status plays a role in the coppermolybdenum-sulfate interaction, the second experiment was designed to study this effect. The 48 rats used in this study were copper depleted by feeding a whole milk powder diet for two weeks prior to placing the animals upon treatment. At the end of the depletion phase, the same treatments as used in the first experiment were added to the milk powder diet. Body weights were recorded weekly; hemoglobin concentrations were determined at the end of the depletion phase and at the third and sixth weeks of the six-week treatment phase. At the end of the study, rats were sacrificed; serum collected; and livers, kidneys, and spleens removed. CPA and copper determinations were made as before.

In the copper-depleted study, there were no differences in growth rates. Hemoglobin concentrations were reduced in all rats at the end of the depletion period (Table IV). Following the addition of copper to the diet, hemoglobin levels began to increase. However, hemoglobin repletion was slower in the Group C, lindgrenite, rats compared with the rats receiving copper sulfate (Groups B and D). The presence of sodium molybdate appeared to have no separate effect (Group D vs Group B). The treatment effects upon CPA and tissue copper in the copper-depleted study are summarized in Table V. As in the earlier study, Group C rats had significantly reduced CPA. One possible explanation for the greater CPA in this study than in the earlier one is that the rats contracted a minor respiratory ailment about the midpoint of the study. This could account for the increased activity since it has been reported that stress conditions do
increase CPA. Even sa, neither the increased CPA nor the amount of the increase was as great in the lindgrenite treated rats as in the other two copper supplemented graups. Cantrary to the data obtained in the previous study, in this copper deplation study there were na significant differences in liver and kidney copper concentratians among any of the copper supplemented groups. Such a difference would not necessarily be expected in the latter study since tissue stares had been depleted prior to treatment. In fact, the lack of a difference in liver copper levels between the lindgrenite supplemented group and the capper sulfate supplemented groups seems to suggest that lindgrenite copper is readily absorbed and taken up by the tissues in rats. The higher spleenic copper cancentration in the Group D rats has no apparent explanation at the present time.

CONCLUSIONS:

Regardless of the capper status of the rat, the data presented herein indicate that capper in the farm of the Cu-Mo compound, synthetic lindgrenite, is not as available for metabalic use, particularly for ceruloplasmin oxidase activity, as is copper from the sulfate salt. Since an amount of molybdenum equal to that in the lindgrenite did not produce effects similar to the lindgrenite, it appears that the reduced availability of capper from lindgrenite is a function of that molecule and not dependent simply upon the presence of molybdenum in the diet.

RECOMMENDATIONS:

Cantinue to study copper metabalism to arrive at a more lucid explanation of its function. These studies are important from the point of view that many af the compounds which interact with copper fall into the category of pollutants; and only when copper functions and mechanisms are known can one make apprapriate strides toward cambating the action of metabolic antagonists.

PUBLICATIONS:

1. Dowdy, R.P. Copper metabolism. Am. J. Clin. Nutr., (In press).

2. Dowdy, R.P., Geo.gia A. Kunz, and H.E. Sauberlich. "Effect of a cappermalybdenum compound upon capper metabolism in the rat." (Submitted far publication). Basic Studies of Nutrition and Metabolism (Cont^a d)

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TABLE I

Composition of Basal Diet

Ingredient	Amount (%)
Vitamin -free casein	20.0
Cerelose	62.2
Alphacei	5.0
Corn oil	5.0
L-cystine	0.3
Mineral-mix ^a	5.0
Vitamin-mix ^b	2.5

^aMineral-mix contained: $(g/kg \text{ diet}) Ca(H_2PO_4)_2$ H₂O, 16.4; CaCO₃, 11.5; KH₂PO₄, 7.6; MgSO₄· 7H₂O, 5.1; Na₂HPO₄·7H₂O, 1.2; NaCl, 1.0; MnSO₄·H₂O, 0.19; FeC₆H₅O₇·5H₂O, 0.18; ZnO, 0.014; KI, 0.00023.

^LVitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

TABLE II

Treatments Added to Basel Diet

Group No.	Additive
Α	Nothing
В	15 mg CuSO _A /kg
С	18 mg Cu-Mo/kg
D	15 mg CuSO _A /kg+
	$16 \text{ mg Na}_2 MoO_4 \cdot 2H_2 O/kg$

TABLE III

Effect of Copper Source on Serum Ceruloplasmin Oxidase Activity and Tissue Copper Concentration*

<u> </u>	Ceruloplasmin		Tissue	
	Activity	Liver	Kidney	Spleen
	(I.U.)	(ppm	on dry weight	basis)
A	1.61 a	6.50 a	11.4ĭ a	1.50 a
В	30.38 Ь	11.93 Ь	31.24 Ь	5.43 b
С	25 .67 c	10.70 c	24.41 c	7.17b
D	29.33 Ь	12.25 b	30.25 Ь	6.06 b

*Each value represents the mean of 12 observations and each value in a column not followed by the same letter is significantly different at the 95% level.

TABLE IV

Effect of Copper Depletion and Repletion on Hemoglobin*

Group	Wee	eks Post-Tre	atment
	0	(g∕ ³ 00 m	6
Α	9.87	10.20 a	9.28 a
В	9.88	13.30 Ь	13.95 b
С	9.83	12.10 c	13.00 c
D	9.59	13.38 Ь	14.05 Ь

* Each value represents the mean of 12 observations and each value in a column not followed by the same letter is significantly different at the 95% leve!.

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TABLE V

Effect of Copper Source following Depletion on Serum Ceruloplasmin Oxidase Activity and Tissue Copper Concentration*

C	Ceruloplasmin		Tissue	
	Activity	Liver	Kidney	Spleen
<u></u>	(I.U.)	(ppm	on dry weight	basis)
A	1.74 a	4.39 c	8.81 a	2.25 a
В	47.16 Ь	9.09 b	32.49 Ь	5.84 b
С	35.13 c	8.64 b	29.81 Ь	5.86 b
D	48.81 Ь	9.17 b	29.53 b	4.52 c

* Each value represents the mean of 12 observations and each value in a column not followed by the same letter is significantly different at the 95% level.

b. Effect of zine deficiency on sulfur-35 and hexosamine metabolism in the epiphyseal plate and primary spongiosa of the chick.

PROBLEM:

In zinc deficiency, chicks fed a soybean protein based diet develop swollen hocks, and shortened and thickened leg bones. The cartilage cells are arranged more randomly in the epiphyseal plate, and the epiphyseal-diaphyseal junction is narrower. Recently, Westmoreland and Hoekstra (J. Nutr. 98, 76 (1969)) reported in detail the pathological defects in the epiphyseal cartilage of zinc deficient chicks. Using histological methods, these workers found that in zincdeficient chicks, areas which were near neither the hyaline cartilage nor the penetrating blood vessels had more matrix, and the cells instead of being uniformly flattened and in regular columns as in normal bone, had irregular shapes and more random locations. In the zinc-deficient chick, the cells of the degenerating region of the epiphyseal plate did not degenerate normally unless they were near a blood vessel. These investigators also reported that no difference in the distribution of either mucopolysaccharides or collagen, the two main components of extracellular matrix, was found. However, in the zinc-deficient chick, cells remote from blood vessels were much delayed in their development of alkaline phosphatase. Zinc-deficient chicks fed histamine or indomethacin showed the same distribution of alkaline phosphatase as did the zinc-deficient controls. Since these compounds grossly moderate the leg defect it appears they may be acting on some other defective metabolism in this area of the bone. Although the distribution of mucopolysaccharides does not appear to be affected, it seems possible that this fraction may be altered in some other manner. High alkaline phosphatase activity has been linked to extracellular matrix secretion and specifically to mucopolysaccharide synthesis. Moreover, antiarthritic compounds which moderate the leg defect in zinc-deficient chicks often affect mucopolysaccharide metabolism. Therefore, experiments were conducted to see if the sulfate sulfur (a major component of mucopolysaccharide in bone) metabolism was altered in some manner in zina-deficient chick bone.

RESULTS AND DISCUSSION OF THE RESULTS:

Two experiments were conducted with day-old White Rock chicks without segregation according to sex. The birds were distributed at random into groups of 15 each and placed in a stainless steel battery at 37°C to 40°C. Distilled water and feed was provided in aluminum troughs. Feed was mixed every two weeks and stored in a refrigerator until it was fed. The composition of the basal soyprotein diet was that described previously (J. Nutr., 94, 527 (1968)). The basal

diet, with 5 ppm supplemental zinc (zinc-deficient diet) analyzed approximately 14 ppm zinc on an air dried basis. To obtain a control diet, 80 ppm zinc as zinc oxide was added to the basal diet. In both experiments, 5 groups of 15 chicks were given 1 of 3 dietary regimes. One group of 75 chicks was given the zincdeficient diet ad libitum. A second group of 75 chicks was given the control diet ad libitum. The final group of 75 chicks was given the control fed so that their average weight gain was approximately that of the zincdeficient chicks.

Radioactive sulfur as $Na_2^{35}SO_4$, carrier free, was diluted with distilled water to give a stock solution of 10 μ Ci of $^{35}S/ml$.

In both experiments, after approximately 4 weeks on diet, the chicks were divided into 2 groups. One group received $10 \mu \text{Ci}$ of 35 S, $1.\text{p}_{\odot}$; the other, $10 \mu \text{Ci}$ of 35 S by gavage. At 6, 12, 24, 48, and 96 hours after isotope administration, 5 chicks from each group were killed by cervical fracture. The legs were removed and frozen until 35 S analyses could be made.

The ³⁵S content of the complete epiphyseal plate through the hypertrophic zone, and the complete primary sponglosa from the proximal end of one tibla from each chick were unalyzed.

The average weights of the chicks used are given in Table 1. In both experiments; the chicks fed the control diet ad libitum weighed approximately 50% more than the chicks fed the zinc-deficient diet. The chicks which were restricted fed weighed slightly more than the zinc-deficient chicks. However, the weights were close enough to insure that the amount of isotope given per unit of weight was approximately the same as that for the zinc-deficient chicks. Table 2 gives the dpm/mg of 35 S found in the fresh epiphyseal plate and Table 3 gives the dpm/mg of 35 S in fresh primary spongiosa during the time periods studied. In the experiment 1 (oral administration), both the restricted fed and ad libitum fed control chicks had significantly higher peak 35 S incorporation in both tissues. The peak level of isotope incorporation occurred at approximately 12 hours after administration. Zinc deficiency also appears to affect the removal of 35 S from the epiphyseal plate and primary spongiosa of the chick. At the 48 hour time period, the chicks fed the control diet had a level of 35 S in the epiphyseal plate equal to that in the zinc-deficient chicks. By the time the 96 hour time period had been reached, the chicks fed the ad libitum control diet had a significantly lower 35 S content in both the epiphyseal plate and primary spongiosa.

The chicks given ³⁵S by 1.p. injection gave essentially the same results as the chicks given the oral administered isotopes as indicated by Tables 2 and 3.

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Although zinc-deficiency decreased 35 S peak retention and removal in the epiphyseal plate and primary sponglosa of the chick, a reduction of the hexo-samines in these areas did not occur. Table 4 shows that the zinc-deficient chicks had a non-significant elevation of hexosamines in the epiphyseal plate, and a slight, but significant, elevation of hexosamines in the primary sponglosa. These results suggest that the metabolism of SO₄, an important constituent of mucopolysaccharide in the area of bone elongation has been altered.

CONCLUSIONSI

The effect of zinc deficiency in the chick on sulfate sulfur metabolism in the area of bons elongation was investigated. Sulfur-35 incorporation into the epiphyseal plate through the hypertrophic zone and the primary sponglosa of tibias was less in the zinc-deficient chicks when compared to the zinc supplemented groups. The results suggest that the metabolism of SO_4^{-} , an important constituent of mucopolysaccharids in the area of bone elongation was altered.

PUBLICATIONS

Nielsen, F.H., R.P. Dowdy, and Z.Z. Ziporin, "Effect of zinc deficiency on sulfur-35 and hexadmine metabolism in the epiphyseal plate and primary sponglase of the chick." (manuscript prepared for submission to the J. of Nutrition).

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TABLE 1

AVERAGE FINAL BODY WEIGHT AND WEIGHT GAIN OF CHICKS

TREATMENT	AVERAGE FINAL BODY WEIGHT*	AVERAGE WEIGHT GAIN
	g.	ğ
	EXPERIMENT 1 (Oral Administration)	
Zinc-deficient	184	146
Zinc-sufficient restricted fed	203	165
Control	313	275
	EXPERIMENT 2 (1.p. Administration)	
Zinc-deficient	183	145
Zinc-sufficient restricted fed	203	165
Control	303	265

*Body weights were taken at 4 weeks.

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TABLE 2

355 CONTENT IN EPIPHYSEAL PLATE AT TIME INTERVALS AFTER A SINGLE ORAL OR 1. p. DOSE

		HOU	IRS AFTER ADN	INISTRATIO	N
TREATMENT	6	12	24	48	96
			dpm/mg fre	sh tissue	
	EXPERIMENT 1 (0	ral administr	ation)		
Zinc-deficient	419 ⁰	545 ⁰	449 ⁰	415 ⁰	163 ^{°°}
Zinc-sufficient restricted fed	873 ^b	1554 ^b	1250 ^b	636 ^b	106 ^{°°}
Control	689 ⁶	1117 ^b	867 ^c	427 ^a	59 ^b
	EXPERIMENT 2 (1	.p. administr	ration)		
Zinc-deficient	698 ^{°°}	604 ⁰	524 ⁰	552 ^{°°}	223 ^{°°}
Zinc-sufficient restricted fed	1352 ^b	1351 ^c	965 ^b	586 ⁰	190°
Control	705 ^{°°}	996 ^b	643 ^{a, b}	574 [°]	72 ^b

a. Mean, 5 chicks/group.

b. Values followed by the same letters within a given time period and experiment are not significantly different (P > 0.05) from each other.

TABLE 3

35 CONTENT IN PRIMARY SPONGIOSA AT TIME INTERVALS

AFTER A SINGLE ORAL OR L.P. DOSE

		HOURS AF	TER ADMINIS	TRATION	
TREATMENT	6	12	24	48	96
		d	lpn√mg fresh t	tissue	
	EXPERIMENT 1 (c	oral administr	ation)		
Zinc-deficient	136 ⁰	169 ⁴	140 ^a	163 ⁰	1190
Zinc-sufficient restricted fed	246 ^b	559 ^b	455 ^b	311 ^b	107 ^{a, b}
Control	256 ^b	369 ⁶	348 ⁶	298 ⁶	73 ^b
	EXPERIMENT 2 (i	.p. administr	ration)		
Zinc-deficient	1400	268 ⁰	259 ^{°°}	296 ^a	152 ^{°°}
Zinc-sufficient restricted fed	266 ⁰	611 ^b	491 ^{0, b}	529 ^{0, b}	191 ^a
Control	186 ^{°°}	411 ^{0, b}	365 ^b	458 ^b	88 ⁶

a. Mean, 5 chicks/group.

b. Values followed by the same letters within a given time period and experiment are not significantly different (P > 0.05) from each other.

TABLE 4

HEXOSAMINE CONTENT IN EPIPHYSEAL PLATE AND

PRIMARY SPONGIOSA FROM CHICKS FED DIFFERENT LEVELS OF ZINC

TREATMENT	DIETARY ZINC	TOTAL H	EXOSAMINE
	ррт	mg/g	fresh tissue
		Epiphyseal Plate	Primary Spongiosa
Zinc-deficient	14	11.54 ⁰	8.61 [°]
Zinc-sufficient restricted fed	90	11.01°	6.86 ^b
Control	90	11.00 ^{°°}	6.84 ^b

a. Mean, 5 chicks/group.

b. Values followed by the same letters within a given time period and experiment are not significantly different (P>0.05) from each other.

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ABSTRACT

PROJECT NO.	3A061102B71P	Basic Research in Support of Military Medicine
TASK NO.	01	Biochemistry
WORK UNIT NO.	061	Mineral Metabolism

Dietary requirements and some of the interrelationships among sex, methionine ingestion, vitamin E and dietary selenium were established for the production of a selenium deficiency syndrome in the rat. Liver necrosis and hemorrhaging develops rapidly after feeding weanling rats a deficient diet for three weeks. Death occurs within three days after cessation of growth and a great reduction of food intake. Selenium analyses of tissues from deficient animals show a large decrease of the selenium contents of blood, kidneys, spleen and liver. Selenium deficiency increases nitrogen excretion which results in negative nitrogen balances. This is indicative of an impairment in protein metabolism.

BODY OF REPORT

WORK UNIT NO. 061

Mineral Metabolism

PROBLEM:

A selenium deficiency affects different organ systems in various species of animals; however, a necrotic degeneration of the tissue results in the susceptible organ, i.e., liver in rats, heart and kidney in mice, gizzard in turkeys, muscle in sheep, and blood vessels in chickens. It has been observed in some children being treated for kwashiokor that a positive nitrogen balance could only be achieved after administering selenium. In these cases, serum levels of selenium were reduced 50% in comparison to unaffected children from the same geographical region. Dietary supplementation with methionine delays the onset of the deficiency syndrome in various experimental animals. All of these observations are consistent with the hypothesis that a selenium deficiency results in a defect in protein metabolism.

The primary objectives of these studies are to test the hypothesis that selenium is required for protein metabolism and if the hypothesis is true, to elucidate the mechanism of action of selenium. The initial studies established dietary conditions required to produce a deficiency, the time required for a deficiency to develop in weanling rats and the selenium levels of various tissues of the animals. The last study was on the effects of selenium deficiency upon nitrogen balance.

RESULTS AND DISCUSSION OF THE RESULTS:

Five studies have been conducted in the past three years using weanling albino rats. Preliminary data from these studies indicated that either vitamin E or selenium would ameliorate the syndrome produced by feeding a torula yeast-stripped lard diet in the rat. Methionine supplementation, in the absence of added selenium and vitamin E, would prolong survival but eventually the animals succumbed to the deficiency. In the male rat, survival appeared to be related to the growth rates since the rats died when they attained a certain weight range. This weight range was elevated as the dietary level of methionine was increased. A method for selenium analyses utilizing the low temperature dry asher and fluorometric determination of the element was established and analyses of tissues have been completed. Preliminary evaluation of these results indicates that the selenium deficiency in the rat increases nitrogen excretion and a negative

Mineral Metabolism

nitrogen balance results. This would indicate that a sclenium deficiency impairs protein metabolism; however, statistical analyses of the data will be required before definitive statements are made.

CONCLUSIONS:

A selenium deficiency in the rat reduced the intake and increased the excretion of nitrogen so that negative nitrogen balances occurred. These observations would indicate an impairment of protein metabolism in the deficient animal. Further statistical analyses of the data are required.

RECOMMENDATIONS:

Depending on the results of the selenium deficient animals, other studies may be designed to further delineate the influence of selenium deficiency on nitrogen metabolism.

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ABSTRACT

PROJECT NO.	3A061102B71P	Basic Research in Support of Military Medicine
TASK NO.	01	Biochemistry
WORK UNIT NO.	062	Haemopoietic Metabolism as Related to Nutrition, Genetics and Metabolic Disease
STUDY NO. 1		To investigate the 1- ¹⁴ C- glucose Metabolism in Red Cells from Germ-free and Pathogen-free rats on Vitamin Deficient Diets.

Abstract. Germ-free and pathogen-free rats have been kept on riboflavin, thiamine and folic acid deficient diets. The metabolism of $1-\frac{14}{4}$ G-glucose has been studied in the red cells from these rats. These vitamin deficiencies do not appear to affect the pathogen-free and germ-free rat red blood cells. The rat red blood cells appear to resist vitamin deficiencies when the pentose phosphate pathway is studied by means of $1-\frac{14}{3}$ G-glucose. In the folic acid deficient rats, at a time when gastrointestinal enzymes were markedly decreased, the rat red blood cells showed no significant impairment of the pentose phosphate pathway. This is of interest since folic acid generates TPN from TPNH which would be of importance in the pentose phosphate pathway. In addition, riboflavin is implicated in the control of the pentose phosphate pathway and a deficiency of riboflavin does not seem to affect the pentose phosphate pathway of the red cell. Similarly, thiamine which is involved in the transketolase step dows not seem to have much effect on the intact red blood cell in the thiamine deficient germ-free rat.

BODY OF REPORT

WORK UNIT NO.

062

STUDY NO. 1

Haemopoietic Metabolism as Related to Nutrition, Genetics and Métabolic Disease

To investigate the 1-¹⁴C-glucose Metabolism in Red Cells From Germ-free and Pathogen-free Rats on Vitamin Deficient Diets.

PROBLEM :

The regulation of metabolic pathways is poorly understood in most tissues, particularly in man. The red blood cell is a convenient tissue in which to study metabolic pathways inasmuch as only certain of the pathways are present and complicating features such as nuclei and mitochondria are absent. Thus, it is convenient to use red cells for the study of the pentose phosphate pathway in an attempt to elucidate the mechanisms of control of this pathway. Since vitamin deficiencies were being studied in germ-free and pathogen-free rate it was ideal to obtain red cells from these animals to study the pentose phosphate pathway under the con-ditions of various vitamin deficiencies. Using 1-14C-glucose one can collect radioactive carbon dioxide and use this as a measure of activity of the pentose phosphate pathway in the red blood cell. The animals were on riboflavin, thismine or folic acid deficient diets. Riboflavin accepts hydrogen from reduced TPN, thus regenerating TPN. It has already been established that riboflavin accelerates the pentose phosphate pathway when added to intact red cells, particularly to red cell hemoiysates. Thismine is an important cofactor in the transketolase step in the pentose phosphate pathway but not directly connected to the production of CO2 or regeneration of TPN. Folic sold is present in red cells and is transformed to tetrahydrofolic acid utilizing TPNH thereby regenerating TPN. Rather large amounts of exogenously added folic acid are necessary to atimulate the pentose phosphate pathway. This may be in part due to a slow uptake of folic acid by intact red cells. Theoretically it is possible, however, for folic acid to be regulated by and to regulate the pentose phosphate pathway by means of the shuttling of TPN between the two pathways.

RESULTS AND DISCUSSION OF THE RESULTS:

Germ-free and pathogen-free rats were placed on riboflavin or thiamine deficient diets. Red cells were obtained and the pentose phosphate pathway was studied by means of the production of ¹⁴CO₂ from }-¹⁴C-glucose. In general, the intact red cells were resistant to the effects of the riboflavin or thiamine deficient diets with regard to the pentose phosphate pathway with and without added cofactors. The intact cells were poorly stimulated by the addition of riboflavin and NADP to the incubation media. Hemolysates could be stimulated but the vitamin deficient state did not seem to have an influence on the degree of stimulation. There

Haemopoietic Metabolism as Related to Nutrition, Genetics and Metabolic Disease (Cont'd)

appeared to be some subtle differences but these did not appear to be physiologically significant. The same resistance was seen in intact red cells from folic acid deficient animals. At a time when the folic acid deficient animals had decreased ensyme activity in the gastrointestinal tract the red cells did not appear to be affected by the folic acid deficient state. The intact cells stimulated poorly to the added cofactors while the hemolysates did respond but the response was not consistently related to the folic acid deficient state.

CONCLUEIONE:

Nee cells obtained from Animals on riboflavin, thiamine or folic acid deficient diets show no change in the pentose phosphate pathway as measured by release of ¹⁴CO₂ on incubation with 1-¹⁴G-glucose. Even though these cofactors are implicated indirectly in the regulation of the pentose phosphate pathway this does not seem to be the case in the vitamin deficient rat red blood cell. We must conclude that the intact rat red blood cell is not the most suitable tissue for the study of this regulatory phenomena. It probably would be more significant to study this in humans. However, it is much more difficult to apply vitamin deficient diets to man than enimals. The resistance of the rat red cells to vitamin deficient diets may be due to a species difference and may not necessarily indicate that these particular cofactors are not important in the regulation of the pentose phosphate pathway.

RECOMMENDATIONS:

It would seem more fruitful to carry out studies involving these cofactors in human red cells and such is being contemplated.

PUBLICATIONS

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2. Herman, Y.F., J.W. NeHugh and N.H. Herman. The 1-¹⁴G-glucose metabolism of red blood cells of germ-free rate on a folic acid deficient diet. Proc. of the 8th Annual Meeting of Association for Cnotobiotics, Oakridge, Tennessee, June 10-13, 1969 (in Press).

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ABSTRACT

PROJECT NO.	3A0611028718	Research in Blomedical Sciences
TASK NO.	02	Internal Medicine
WORK UNIT NO.	055	Experimental Surgery in Support of Medical Research

During the past year, surgical support for the following USAMRNL projects has been given: (1) cardiovascular and hematological effects of prolonged starvation followed by refeeding in swine; (2) determination of the ethology of canlne enostosis; (3) effects of coronary sinus stretch receptors in remai function; (4) the metabolic effects of glucagon and adrenaline in intact and pancreatectomized geese and dogs; and (5) the effects of high altitude and hypoxia on the cerebrospinal fluid pressure in dogs.

Surgical support for research conducted by personnel of Fitzsimons General Hospital has consisted of: (1) the feasibility of canine limb replantation and transplantation, (2) the feasibility of using pericardium for large venous grafts, and (3) the effect of lactated Ringer's solution to 30%, 40% and 60% bled dogs.

The surgical facilities of USAMRNL have been used by approximately 40 Fitzsimons General Hospital surgical residents and interns during the past year. Also 80 clinical specialist trainees have received training in suturing techniques of wounds and lacerations during the past year.

BODY OF REPORT

WORK UNIT NO. 055

Experimental Surgery in Support of Medical Research

PROBLEM:

The objectives of this work unit have continued to be primarily twofold: (1) Surgical support for physiological and metabolic studies and; (2) Surgical support of clinical research and teaching activities by Fitzsimons General Hospital personnel.

RESULTS AND DISCUSSION:

A study to determine some cardiovascular and hematological effects of prolonged starvation followed by refeeding in swine was supported. Heart rate, arterial and pulmonary arterial blood pressure were measured, using indwelling catheters in swine that had been starved 28 days and refed 16 days. The heart rate and arterial pressure decreased below normal during starvation and increased to above normal during refeeding. Pulmonary hypertension developed during refeeding.

Canine cosinophilic panosteitis or enostosis was studied. This discase was followed radiographically for six months during the acute, inactive and healing phases. Extensive bacterial, mycoplasma and viral culture studies were performed. Bone marrow specimens contained a <u>Corynebacterium spp.</u>; evidence of mycoplasma or viral infection was not found.

Surgical assistance is being given to a study to determine the effects of coronary sinus stretch receptors on renal function.

Surgical assistance is being given to a project designed to study the metabolic effects of glucagon and adrenaline in intact and pancreaectomized geese and dogs.

The effects of high altitude (14,000 - 16,000 ft.) and hypoxia on cerebrospinal fluid (CSF) pressure in dogs are being studied. Anesthetized dogs, with indwelling catheters in the subarachnoid space, placed in an altitude chamber are being utilized. Preliminary data at this time indicates a probable tenfold increase in CSF pressure at 14,000 - 16,000 feet.

A project to determine the feasibility of limb replantation with and without preservation is being supported. The project is under the direction of Fitzsimons General Hospital personnel. At the present

Experimental Surgery in Support of Medical Research (Cont⁴d)

time, no surviving limbs have been obtained using hyperbaric oxygenation as the means of preservation. Transplantation of canine limbs has also been tried. The use of antilymphocyte serum, Immuran^B, and corticosteroid drugs has allowed some of the limbs to survive up to 60 - 90 days before they were rejected.

A pilot project from Fitzsimons General Hospital to determine the feasibility of using pericardium as a venous graft in the anterior vena cava of dogs is being supported. Approximately 25 dogs have been operated. Ninety day survivors have been obtained before the grafts have become occluded.

A project from Fitzsimons General Hospital to determine the effect of hemodilution on cardiac output, cardiac oxygen utilization and tissue oxygenation following hemorrhage was supported. Thirty splenectomized dogs were bled 30%, 40% and 60% of their estimated blood volume. Three times the volume of shed blood was replaced with lactated Ringer's solution. The following measurements were made: hematocrit, cardiac output, oxygen tension of cerebrospinal fluid, urine, blood in the coronary sinus, arterial blood and systolic and diastolic blood pressure. Although this study has been completed, the data have not been made available to the collaborating staff of this laboratory.

The surgical facilities at USAMRNL are also being used by Fitzsimons General Hospital personnel for surgical training of interns and residents. Approximately 40 Fitzsimons General Hospital surgical residents and interns utilized the laboratory surgical facilities during the past year. Finally, a surgical training program for students of the Clinical Specialists' Training Program at Fitzsimons General Hospital is being conducted. During the past year, 80 clinical specialist trainees have received training in suturing techniques of lacerations and wounds.

CONCLUSIONS:

Surgical support for physiological and metabolic studies by USAMRNL staff is being provided. New surgical techniques and improvement of standard techniques are being sought. Surgical support to research programs carried on by Fitzsimons General Hospital is being given. Student training has been successful and will continue.

Experimental Surgery in Support of Medical Research (Contⁱd)

PUBLICATIONS:

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ABSTRACT

PROJECT NO. 3A061102871R

TASK NO. 02

WORK UNIT NO. 057

Research in Biomedical Sciences

Internal Medicine

Maintenance of Animals and Study of Pathology of Animals Utilized in Research

This work unit covers the division's studies in diseases of laboratory animals, the maintenance of animals used by all divisions, the conduct of a training program in veterinary pathology and the provision of pathology services to other laboratory divisions.

Seven hundred twenty-six cases, including 561 necropsies, were accessioned. These resulted in 5,342 tissue blocks, more than 7,000 H&E slides, more than 1,100 specially-stained slides, and a few thousand unstained slides. These materials are used in the Veterinary Pathology Preceptorship Program in addition to supporting case studies and publications.

About 4,600 animals were reared or obtained from outside sources. Included were 2,882 rats, 420 mice, 290 guinea pigs, 216 dogs, 600 chickens, 80 rabbits, 30 monkeys and approximately 50 each of turkeys, gerbils, squirrels and a small number of tree-shrews, caimans and iguanas.

The total numbers of animals used and of tissue processed are somewhat less than FY 68 (except 80% increase in number of dogs used, 30% increase in number of mice and several-fold increase in number of chicks). The decrease reflects the laboratory-wide personnel shortages for the year, as well as a possible trend away from morphologic methods and in vivo studies. Pathology Division generates most of the requirements for tissue processing. Technician shortages have required curtailment of microslide production.

In vivo phases of studies carried over from FY 68, involving investigations of spontaneous disease of animals, were completed. For most of these the tissues are not completely processed or the slides read and the dato tabulated (Myopathology in Turkeys, Steatitis in Caimans, Staphylococcus in Rabbits).

BUDY OF REPORT

WORK UNIT NO. 057

Maintenance of Animals and Study of Pathology of Animals Utilized in Research

PROBLEM:

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1. To detect, diagnose and study disease occurring in this laboratory's animal colony and to initiate studies designed to elucidate the nature, pathogenesis and pathology of diseases of laboratory animals.

2. To maintain animals used by all divisions of the laboratory.

3. To provide pathology service to the laboratory. This function also supplies case material and support for the Veterinary Pathology Preceptorship Program, a program designed to train career VC officers in animal pathology.

RESULTS AND DISCUSSION OF RESULTS:

1. As in previous years, spontaneous disease was rare in the animal colony. This is attributed to careful husbandry, careful selection of animal suppliers and the nature of the studies, which require younger animals, primarily. There were a small number of rabbits purchased with hepatic coccidiosis, some with ear mites and some rats with chronic murine pneumonia, but the total number was very small. Dags from a local municipal pound occasionally develop respiratory disease or enteritis during the first few days of conditioning but most respond to therapy. Those which do not respond usually have canine distemper and ore euthanatized.

2. Diagnosis of Tyzzer's Disease: One shipment of young rats contained a number of animals which tailed to grow normally prior to being placed on experiment. The "poor doers" were sacrificed and several had focal hepatic necrosis and slightly distended intestines. In one such rat, typical intracytoplasmic bacteria (Bacillus piliformis) were present in hepatocytes and intestinal epithelium. These organisms, which cannot be grown on artificial media, were not found in the other similarly-affected animals. This is opparently the first recorded incident of this disease occurring spontaneously in rats. At presentation of this case at the 1969 meeting of the International Academy of Pathology, an investigator

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from Yale University School of Medicine commented that he had experienced a similar autoreak, but in rats already on experiment. Our case is wring readied for publication.

3. Pothology Service.

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a. Light microscopy.

In addition to intradivisional research, input includes submissions from investigators of other USAMRINL Divisions, some from Clinical Research Service, Fitzsimons General Hospital and special cases solicited from the Denver Zoological Park, private veterinary practitioners and the Animal Service of the University of Colorado Medical Center. The special cases provide varied species and disease conditions for the training program.

Seven hundred twenty-six cases, including 561 necropsies were accessioned. these resulted in 5,342 tissue blocks, more than 7,000 slides stained with hematoxylin and easin, more than 1,100 stained with special stains and approximately 7,000 unstained slides. The decrease in this output when compared to FY 68 is attributed to the overall USAMRINL personnel shortages as well as possible trends away from morphologic methods of investigation. Further, Pathology Division generates the largest requirement for tissue processing; overhead microslide production has been curtailed by technician shortage.

While the quality of microslides produced is generally very good, there is a backlog of approximately 100 cases at this time. This results from one civilian (GS-7) position which was vacant for the last 11 months of FY 68 due to the hiring freeze and actual strength of 1-3 enlisted technicians for the year when six are authorized and required.

b. Electron Microscopy.

This service has been performed by an MSC electron microscopist with the aid of an equivalent of 1 technician (2 part-time). In addition to Work Unit 054, Ultrastructure of Animal Tissue, which is reported separately, ultrastructural studies have been carried out collaboratively with USAMRINL divisions, with several services of Fitzsimons General Hospital and with the National Jewish Hospital, Denver. There were approximately 160 electron microscope assessions under the 057 work unit, resulting in some 220 photographic plates.

Maintenance of Animals and Rudy of Pathology of Animals Utilized in Research (Contid)

Innumerable apportunities for electron microscopic examination of changes noted with the light microscope had to be overlooked because of shortage of technical help. Similarly, there is a vast unexplored area in ultrashiucturacharacterization of tissue of normal laboratory animals. The normal ultrashiucture must be defined as a basis for evaluation of change produced by experimentation. These important and productive areas now go unexplored for lack of sufficient technical help.

c. Autorodiography.

A collaborative study with the University of Colorado Chemistry Department on Vitamin C generated approximately 150 microslides which were coated with photographic emulsion and will be examined for cellular location of tritiated ascorbic acids on 2 and 4-micron thick frozen tissue sections.

d. Clinical pathology.

Procedures performed were in support of research projects and the animal colony. During the reporting period the following were performed: 319 white blood cell counts, 212 packed red cell volumes, 114 hemoglobins, 49 red blood cell counts, 296 reticularyte counts, 25 sedimentation rates, 275 differentials, and 247 other blood determinations including BUN, enzymes, glucose, cholesterol; others included determinations of 69 urinallyses and 180 fecall examinations. The smaller number of analyses performed reflects the reduced number of animals utilized by the laboratory, as well as the shortage of technicians: while more frequent followups may have been desirable in certain cases, most optional determinations were eliminated. When tests were considered important but staffing was inadequate, Pathology Service, FGHt kindly performed many determinations. During one field study (Work Unit 061), one technician was on TDY for 3 months of FY 68 and performed many laboratory procedures.

e. Thin-Section microscopy.

Thin-section microscopy, in which tissue sections are fixed and embedded by techniques suitable for electron microscopy, and cut at 0.5 to 2 microns thickness, provides for elegant morphologic analysis. The merits of this technique and our hopes to expand its use are described in the Annual Report for FY 1988. During the second quarter of FY 70, necessary laboratory space will be available and we shall begin to develop methods at that time.

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Mointenence of Animals and Study of Participagy of Animals of Lates in Research (Cont 6)

4. Animal Service.

Animals and purchased, housed and feet for investigators of all VRNAL stations and for Clinical Research Service, FOrt. A total of approximately 4,500 animals were abtained from outside sources or reared. This number includes 2,332 -or., 400 mice, 200 guined pigs, 200 dogs, 600 chickens, 90 naturity, 30 munkelys. approximately 50 each of gentalis and turkelys and a few each of ground squine s, the shrews, calmans and iguanas. These numbers are somewhat lower than those for FY 68, for the reasons cited under Pathology Service, including a cycle of investigative interests not so heavily dependent upon animal use as in previous years. The number of dogs used did increase by 80%, however, and mise and chicks were also used in greater number.

Facilities for all animals except dogs are sortisfactory, ten of the 30 monkeys are housed on MARINE, the first time this species has been a single room is set aside for their use, precluding quarantine of additional monkeys until the present group is socificed.

Dog facilities continue to be inadequate. We are utilizing dogs currently or 3-10 per week, with turnover at approximately 2-3 weeks for more than had the dogs. Adequate quarantine facilities do not exist. Physical segregation is attempted. Oversized cages to accommodate 50-1100 dogs are in use, further cramping the present insufficient space. Fifteen to 20 dogs (bill those in excess of our 25-35 dog capecity) were boarded commercially at a nearby kennell, at a cost of \$6,400 for FY 69.

Animal service allow includes the feeding of diets to experimental animals. Such diets are frequently compounded by Animal Service, to the prescription of the investigator and fed as scheduled, with records kept of amount consumed, animal weight, etc. Thirty-two such diets were compounded during fit 67. Several were fed simultaneously for different studies.

3. The Veterinary Pathology Proceptorship Program, described in the FY 33 Annual Report, is a formall, SCO-recognized training program. The USA MRIVIL Pathology Division has two proceptors who are progressing satisfactorially. The structural partian of the program consists of daily T-hour semimors presented by the trainast (all VC officers participate, not just the proceptors) and a weekly slide conference. The schedule is drawn by the Division Chief, who is Preceptor. Training materials consist of all pathology cases and past case filles, to include climical antimation, climical pathologic exam, necropsy and electron microscopy.

No memorice of Animoly and Shidy of Family system works (Filliged 19). Research Control

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a. Wysam closer in Turkers: This shall not described in the Fr SB Konud אי אינייש ישנאי גיגעים יניאי ינטאי גע אני איני איניש אייז אוואי אייאל עיר אנוזיאיט אני איניגענט לאיני אייאי אי mysporty encountered in major includence into turkey filocial. The discose could not be prevented by parenteral supplementation with the land decapterally selections or se en ium-reason to the For Sty groups at attack were chall enged write any appliances or additions on their products, to recreate the disease. Endotoxing and exproving of these proprisms have been described, and we cultured these proprisms repeatedly from the source filocity. The mysparity appeared to be more severe in brinds forcing. mycophisma infection, allthough the infection could have been a result of their debilling norther than a cause. In time groups of 10 studs, administration of live organisms or extracts of cultures, containing potential exotoring endorsion or both, vito muscle or machery, failled to enhance the disease process climically. The binds were exposed repeatedly during their first few weeks of little. At mecropsy of 23-26 weeks, recovery of the mycophisma organisms not common, from both control and test animals. Cultures were send to the U.S. Communicable Discose Center, which reported this exceptional as servicesily untradule.

Tobularium and colliarium of the data nos been deferred until the tissues are examined microscopically.

b. Felline Infectious Peritonitis

As described in the FY SB Annual Report, we rad successfully transmitted this disease is cars, using fresh ascittic filuid from affected cars, but not after the filuid had been flozen. Other investigators had effected transmission with cellfree filtrates of ascittic filuid. We had hoped to describe a virus as cause of the disease; this has yet to be done, allthough virus-like particles take the number reported in some affected cars. In collisionative studies with the Microbiology Division, one isolant produced cytopathic effected raw cells. Potentially indectious material (from spontaneously affected cars) is not presently available. Other attempts at isolation of the effologic agent must await simultaneous availability of both an affected car, potentially susceptible kittens, or cell cultures.

Nationarce of Animals and Shudy of Parkalogy of Animals United in Research (Contral)

 Folle of Vitamini E and Unsaturated Fatty Action in Production on Graphics in Colomans

At the close of FV 68 the calmars in the beet diet group continues in the beet diet group continues in the melt group plateback and then even lose weight difficult these in the smelt group plateback and the lost 2-wook pariod. One of the smelt group died by an and the state and the smelt group is the smelt group at the smelt group is the smelt group at the smelt group at

During FY 59 more of the small group died of pneumonia and did not nove the expected lexions of stantitis and for necrosis. In an artempt to precipitate the synchrome, one year after initiation of the study, the diet of the small group was changed to mackenal, which is higher in unsaturated for content, lagoroximately 20% compared with 7% for smalt). As the caimans on the fish diet died, some of the caimans on the mean diet were transferred to the fish group. After the transferred to the fish group. After the transferred or they lost weight. At the end of the feeding trial only two caimans survived, one in each group, and they were socialized.

Complete necropsies were performed on all the calmens and most of the discuss have been processed for histoparthological examination but the examination has not been completed. Source samples for tocopheroil and fatty acid determinations, adipose tissue biopsies for fatty acids and blood smears for red cell monshology were obtained intermittently and are awaiting analysis.

The consistently-formed lesions of granulamatous pneumonia in the fish diet group with isolation of Reudonas or Proteus organisms from the lesions bear further attention. Perhaps the dietary regime of the calmans in the fish group reduced their resistance to infection. The fish themselves may have introduced a large number of these gram-negative organisms into the tank water of the fish group. The feeding technique may have been contributing. The fish were thawed before feeding and then put in the tank; 24 hours later the tanks were cleaned and the water was changed. The same cleaning technique was used on the tank of the meat group (chopped lean beet stew meat). It was hoped that the cleaning and water changing after feeding (twice weekly) would minimize contamination of the water, but short-term contamination was unavaidable due to the eating tabits of the calmans.

No more animals are scheduled for feeding trials under this study. The specimens obtained must be processed with analysis of the resultant data.

Maintenance of Animals and Study of Pathology of Animals Utilized in Research (Cont'd)

d. Staphylococcal Infection in Rabbits

Two female Californian rabbits were on hand as a carry-over of the work of the previous year. Both rabbits had chronic staphylococcal infections with various dermal, ocular and external genital lesions, usually purulent. Previously, litters had been lost due to staphylococcal infection passed to the sucklings by these does.

We planned to rebreed the two does, treat one with antibiotics to which the organism is susceptible and obtain cultures from the doe during and after kindling and from the young after kindling.

Our plan was thwarted by failure of the does to conceive again. In one doe, age (3 yrs.) may have been a factor. It is quite possible that the external genital infection was occompanied by an internal genital infection or that the systemic effects of the infection prevented conception. Treatment with antibiotics to which the organism was susceptible was instigated on two occasions to retard but not eliminate the infection.

The rabbits were sacrificed and necropsied in March and May. Extensive superficial supportative lesions were seen in both and in one an enlarged, nodular, multicolored liver was seen. The tissues have not been processed for microscopic examination. Maintenance of Animals and Study of Pathology of Animals Utilized in Research (Cont'd)

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AGENCY ACCESSION A DATE OF SUMMARY REPORT CONTROL SYNDOL RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY DA OA 6321 69 07 01 DD-DR&E(AR)6.16 S. DATE PREV SUMAY 4. KIND OF SUMMARY Sh SPECIFIC DATA-SUMMARY SCTY" IS. WORK SECURIT REGRADING DISE'R IRSTR'N S. LEVEL OF SUM 68 07 01 D Change NA NL U U O YES <u>но</u> A WORK LINET 10. NO. /CODES:* PROGRAM ELEMENT PROJECT NUMBER TASK AREA NUMBER WORK UNIT NUMBER 61102A 3AC61102B71R 02 058 B. PRIMARY 3A014501B71R . CONTRIBUTING 61145011 02 CDOG 1412 C. CORTRIBUTING 1. TITLE (Placeda with (U) Nutritional and Metabolic Adaptations and Interrelationships (06) 12. SCIENTIFIC AND TECHNOLOGICAL AREAS 002300 Biochemistry; 002600 Biology; 012900 Physiology S. START DATE 4. ESTIMATED COMPLETION OATE S. FUNDING AGENCY 16 PERFORMANCE METHOD 65 05 CONT DA C In-House IT. CONTRACT/ GRANT IS. RESOURCES ESTIMATE & PROFEESIONAL MAN YRS L. FURDS (In thousands) RECTORN. A DATES/EFFECTINOL Applicable EXPIRATION 69 1.3 40 b. RUMBER-* FISCAL CURNEN & TYPE YSAR & AMOUNT: 70 1.6 67 1.CUM. AMT & KIND OF AWARD: 19. RESPONSIBLE DOD ORGANIZATION . PERFORMING ORGANIZATI **Chemistry** Division HAME! US Army Med Rsch & Nutr Lab -US Army Med Rsch & Nutr La Fitzsimons General Hospital ADDRESS:* ADORESS!* Fitzsimons General Hospital Denver, Colorado 80240 Denver, Colorado 80240 RINCIPAL INVESTIGA Sauberlich, H. E. NAME:* RESPONSIBLE INDIVIDUAL Canham, J. E., COL 303 366 5311 X24214 NAME: TELEPHONE: 303 366 5311 X21108 TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER: 21. GENERAL USE SOCIATE INVESTIGATOR Dohm, Gerald L., CPT, MSC Foreign Intelligence not Considered -DA NAME 12. KEYWORDS (Precede EACH with Security Cloudification Code) (U) Periodicity of eating; (U) Meal-Eating; (U) Nibbling; (U) Adaptation: (U) Enzymes: (U) Lipogenesis: (U) Fatty Acid: (U) Carbohydrates TECHNICAL ODIECTIVE,* 24 APPROACH, 25. PROGRESS (Pumilah Individual paregraphie identified by number, precede test of each with Security Classification Code.) 23. (U) Tech Objective: To determine the mechanisms and significance of biochemica adaptations in the mammal exposed to varied patterns and levels of macronutrient and micronutrlent intakes. To obtain information about dietary and environmental factors that may affect changes in proteins, lipids and minerals in relation to age. 24. (U) Approach: To induce adaptive changes, primarily by dietary means, and to elucidate the biochemical basis for such changes. Included will be studies on the effects of meal-eating, level and type of dietary fat, carbohydrate and protein on gluconeogenesis. Subcellular fractionation of hepatic and other tissues will be conducted and various enzymes quantitated. 25, (U) Progress (Jul 68-Jun 69) Investigations were continued on the metabolism of saturated and unsaturated long-chain fatty acids. ¹⁴C-labeled fatty acids were administered to intact rats, muscle homogenates and lysed mitochondria. Methylmalonate, succinate and CO₂ were isolated and carbon-14 determined. Incorporation of U-14C-linoleate into methylmalonate in vitro was 20 times greater than from U-¹⁴C-palmitate. Rats fed 20% corn oil grew more slowly on vitamin B_{12} deficient than vitamin B_{12} sufficient diets. Biotin and vitamin B_{12} deficiencies were found to decrease the in vivo metabolism of linoleste. The data suggest that one pathway of linoleate oxidation has methylmalonate as an intermediate.

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ABSTRACT

PROJECT NO.	3A061102B71R	Research in Biomedical Science
TASK NO.	02	Internal Medicine
WORK UNIT NO.	058	Nutritional and Metabolic Adaptations and Interrelationship

The following investigations have been conducted under this work unit:

STUDY NO. 1 Effect of vitamin B₆ deficiency and meal-eating on gluconeogenic and lipogenic activities in the rat.

STUDY NO. 2 Bio-oxidation of linoleate via gamma-cleavage.

1. The inability to statistically duplicate the previously observed inhibition of gluconeogenic activity in liver slices associated with B₆ deficiency, was attributed to change in incubation medium to one which diminishes the rate of glycogenolysis (Hastings, et al. 1952). This medium supported greater lipogenic activity than the classical Krebs-Ringer bicarbonate buffer. Otherwise, similar results were obtained.

2. The increase in gluconeogenic activity in meal-fed rats was inversely related to their ability to maintain glycogen stores during starvation. Feeding a diet containing 40 per cent fat abolished the hypercholesterogenic response to meal-eating (5 or 10 per cent fat).

3. A simple basal diet was designed for the study of B_{12} and biotin deficiencies. Inhibition of linoleate oxidation by B_{12} deficiency and correlation of rate of propionate metabolism with rate of linoleate oxidation is further indirect evidence for the existence of the gamma-cleavage pathway for linoleate oxidation.

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BODY OF REPORT

WORK UNIT NO. 058

STUDY NO. 1

Nutritional and Metabolic Adaptations and Interrelationships

Effect of vitamin B₆ deficiency and meal-eating on gluconeogenic and lipogenic activities of the rat.

PROBLEM:

An additional experiment was needed to confirm the hypothesis that aspartate aminotransferase plays a direct role in the dicarboxylic acid shuttle. Since pair-fed controls for the study of B₆ deficiency are also meal-eaters, the effect of meal-eating on liver slice metabolism was also studied.

RESULTS AND DISCUSSION OF THE RESULTS:

The experimental procedure was modified to incorporate a medium that contains a greater concentration of K^+ , HCO_3 and Ca^{++} ions than Krebs-Ringer bica bonate buffer and that supports greater glycogenic activity. Interestingly, the former also supported greater lipogenic activity from pyruvate. B₆ deficient rats showed an increased hepatic lipogenic activity as reported previously, but depression of incorporation of pyruvate-1- or -2-¹⁴C into glycogen was not statistically duplicated.

Consideration of the results from tissue incubations from the "control" animals suggested that food restriction of a diet containing 40 per cent fat did not affect in vitro metabolic activities. In a separate experiment it was established that the decreased incorporation of pyruvate into glycogen displayed by liver slices from meal-fed rats was inversely related to their ability to maintain high glycogen stores. The disappearance of the hyperlipogenic response to meal-feeding by changing to a40 per cent fat diet (Leveille, 1967 and 1968) was extended to a similar effect on the hypercholesterogenic response.

The above is based on completion of data acquisition and analysis of data from studies of previous fiscal years. Due to loss of key personnel, without replacement, no new research was initiated under this study in this fiscal year.

CONCLUSIONS (and RECOMMENDATIONS):

Further study is necessary to define optimum in vitro conditions for tissue slice incubations. In addition, the interactions between control of glycogen and

Nutritional and Metabolic Adaptations and Interrelationships (Cont'd)

lipid storage must be clarified before the primary effects of B_{δ} deficiency and meal-eating can be established. Initiation of studies awaits the availability of proper personnel.

PUBLICATIONS: None

STUDY NO. 2

Bic-oxidation on linoleate via gamma-cleavage,

PROBLEM:

To establish the existence of direct hydrolytic cleavage of the 9 - 10 double bound of linoleate (gamma-cleavage) it was deemed important to demonstrate an inhibition of linoleate oxidation with B_{12} and/or biotin deficiency. These vitamins are required for propionate metabolism—the product of the above cleavage. Studies were also designed to determine the tissue specifities of propionate metabolism in the rat.

RESULTS AND DISCUSSION OF THE RESULTS:

Rats were fed a casein-sucrose basal diet containing 20% corn oil, 0.5% sulfathiazole and sufficient methyl-group donors. Removal of biotin or B_{12} caused growth retardation which was additive when both were removed. This is the first known report on the study of the two deficiencies utilizing such an uncomplicated basal diet. Feeding 2% sodium propionate accentuated the growth depressions.

Hypotonic tissue homogenates were incubated in medias containing $^{14}CO_2$ and propionate of U-14C-linoleate. Heart homogenates fixed CO_2 into acid-stable compounds and oxidized linoleate 5-10x liver homogenates. Rate of CO_2 fixation by kidney was slightly less than by heart, and skeletal muscle possessed very low activity. The capability of heart to metabolize propionate was interpreted to reflect its ability to also metabolize linoleate via gamma-cleavage. B12 deficiency depressed and biotin deficiency enhanced linoleate oxidation by heart homogenates. The latter finding has not been explained.

CONCLUSIONS:

The capability of a specific tissue (exception: skeletal muscle) to metabolize propionate was directly related to its ability to oxidize linoleate. This finding

Nutritional and Metabolic Adaptations and Interrelationships (Cont'd)

plus the inhibition of cardiac oxidation of linoleate by B_{12} deficiency indirectly supports the existence of propiogenic oxidation of linoleate by gamma-cleavage.

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ABSTRACT

PROJECT.	34061102B71R	Research in Biomedical Science
task no.	0Z	Internal Medicine
work unit no,	061	Work Performance and Body Composition as Related to Environment and Nutritional Status

1. BODY COMPOSITION:

The assessment of that most widely variable aspect of body composition, body lat, still proves as elusive as does the gas-free body volume. Complete radioisotopy dilution techniques are well adapted and accepted for the clinical patient as described by Moore (1963), but time and method requirements may not lead these techniques to survey-type studies. Application of these techniques to normal soldiers warrant investigations in the future, when human subjects can be exposed to various stresses resulting in physiological changes. High carbohydrate liquid diets have proven beneficial to humans abruptly exposed to 4300 meters. The estimated changes in body lat and dry protein compartments did not approximate body weight loss, suggesting that body water was also decreased in these subjects. In a 1968 study of men who had exercised stremously and maintained a normal intake at an altitude of 4300 meters, there was a significant decrease in total body water, secondary to a reduction of intracellular water, while the extracellular water remained unchanged. Under these conditions, it appears that there is a loss of total body water during acute exposure to altitude.

BODY OF REPORT

WORK UNIT NO. 061

Work Performance and Body Composition as Related to Environmental and Mutritional Status

PROBLEM:

The vertebrate body, consisting of water, lat, mineral and protein, is in a dynamic state easily altered by the internal and external environment. No single technique of accurately estimating human hody inc. partments exists, yet several methods for approximating any one compartment are available. It is of continuing necessity to seek simple. accurate, and reproducible methods to measure all body compositional aspects. Present investigation includes further verification and application of a simple body volume measurement by water displacement, with adequate correction for contained air and gas, whereby the "residual mass" (body less its bone mineral, water, and lat) represents an active metabolizing mass principally composed of muscle tissue. Into active metabolizing mass will provide a real basis for correlation to various physiological functions. Total body polassium is now being counted using a Nal crystal to compare body potassium as an index of "active metabolizing mass" with estimates as calculated from body volumes and selected anthropometry. Total body water, using deuterium oxide dilution and the analyses by the gas chromatograph, are being determined with some success. Deuterium concentrations in blood, urine and saliva are being compared to determine the validity of the technique. Freezing point depression of body fluids by deuterium oxide may also be a promising technique for estimating total body water.

These methodological problems can be evaluated in conjunction with nutrition and field surveys as well as in studies designed to test the effects of environmental stress (such as heat, cold and altitude) on the human body. It is essential to develop some common denominator that is applicable to all human body types so as to determine the effects of aging as it may be related to other physiologic functions (i.e., energy expenditure and oxygen uptake).

RESULTS AND DISCUSSION OF THE RESULTS:

The 1968 altitude study was designed to test the effects of two diets (one with a normal nutrient composition, and a second high in carbo-

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hydrate and low in fat) on humans abruptly exposed to altitude (4900 Meters). Half of each diet was in liquid form, which had to be consumed before the remainder of the meal (in solid form) was received. The subjects exercised strengously each day. Body weight lockes were moderate. Total body water was significantly reduced by 2.25 kg and intracellular water (from sodium thiocyanate dilution) was noted to have decreased by 3.25 kg while extracellular water was practically insultered. Under these conditions, it appeared that must of the body weight loss was due to hypohydration.

a. <u>Caloric Restriction</u>: Body composition data includes anthropometric, densitometric and isotopic measurements. As expected, following caloric restriction, there were decrements in skinloid thickness and encounterences of arms, waist and gluteal regions, and substantial losses of body protein, but and water. These changes were less pronounced than those observed following total food abstinence (Am. J. Clin. Nutr. 21: 87, 1968). It is anticipated that prepared manuscripts will be forwarded for publication in the immediate intere depicting the findings of the 429 and 500 Cal/day studies. The 1968 Panama study on caloric restriction included measurements of body composition. These data are currently being compiled and evaluated.

b. Methodology:

Methodology for measuring total body water using deuterium oxide as a tracer by gas chromatography has been utilized at this laboratory. Reinvestigation of the method by determining the DO levels in blood serum or plasma at timed intervals of 3, 4 and 5 hours and extrapolating to zero time, has been shown to be the most accurate of all techniques studied. The colligative technique of measuring the number of molecules of varied concentrations of D₂O in body fluids as demonstrated by freezing point depression may be an advance for deuterium analysis. This technique will be compared with the gas chromatographic method of analyzing deuterium oxide. It will be of interest to define alterations in total body water with environmental stress using these techniques.

A shadow shield whole body potassium-40 counter has been purchased and is waiting for temporary installation pending completion of a permanent facility. A moveable sled of variable velocity to allow for different counting times (the subject is scanned from head to toe) is being constructed. Installation and calibration of the equipment in a temporary site should be completed in six months. The counter is unique in its portability and should be well utilized in Work Performance and Body Composition as Related to Economic standard Norvitional Status (Confd)

inture studies, for example, to estimate muscle mass under various experimental and pathological conditions.

c. Nutrition Surveys: Computerization of the survey data to relate energy expenditure and body composition of 196 soldiers in eight age groups (subsisting on standard Army rations) has shown that forced vital capacity decreased with age while residual lung volumes were increased; however, maximal performance and work times decreased with age, as did heart rate and oxygen uplakes. As reported earlier, total body fat increased with age and was not body weight dependent; but was again demonstrated in increased skinfold thismess and body waist circumference.

CONCLUSIONS:

In the 1968 altitude study, under conditions of normal food intakes at altitude, body weights were reduced. Total body water was also significantly reduced at altitude and ICW was also decreased, while ECW was practically unaltered. Under these conditions, it appeared that most of the body weight loss was due to hypohydration. Manuscripts on caloric restriction are being prepared for near future publications.

RECOMMENDATIONS:

Completion and publication of data from the previously mentioned militar mutrition surveys, caloric restriction, and high altitude studies.

Standardization and application of whole body potassium-40 counter in the investigation of muscle mass and exchangeable potassium in normal human subjects ingesting various diets, and in patients with metabolic or muscular disorders with and without drug therapy.

Evaluate body compartment measurements made during environmental and mitritional stress in a study conducted in Panama, September-October 1968.

Work Performance and Body Composition as Related to Environment and Nutritional Status (Cont⁴d)

PUBLICATIONS:

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Apphilician in the feature of

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- Krzywicki, H.J. and C.F. Consolazio. Body composition methodology in military nutrition surveys. Proc. of NAS, National Academy of Sciences, Publication #1598, Body Composition in Man and Animals, 1968.
- Krzywicki, H.J., C.F. Consolazio, L.O. Matoush, H.L. Johnson and R.A. Barnhart. Body composition changes during exposure to altitude. Fed. Proc. 28: 1190-1194, 1969.
 - Krzywicki, H.J., C.F. Consolazio, H.L. Johnson and W. C. Nielsen, Jr. Changes in body water compartments at high altitude. Fed. Proc. 28: 656, 1969. (Abstract)

ABSTRACT

PROJECT NO.	3A061102B71R	Research in Biomedical Science
TASK NO.	02	Internal Medicine
WORK UNIT NO.	061	Work Performance and Body Composition as Related to Environment and Nutritional Status

II. WORK PERFORMANCE:

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The physiology of work and exercise in man is being studied to ascertain methods of measuring and improving the performance and fitness of all ages of soldiers working in extreme environments of heat, cold and altitude. Methodology developed includes:

The continuous measurement of heart and respiration rates, ventilation volume, oxygen uptake during a steady state or maximal work on the bicycle ergometer, and the motor driven treadmill on humans.

The repetitive measurements of cardiac output, arterial and venous pressures, blood gas tensions and pH and other physiological and biochemical parameters in chronically catheterized, treadmillexercised dogs.

Data is now being analyzed from nutrition survey studies relating age and body composition to respiratory function and smoking habits, and bicycle and treadmill maximal work performance on enlisted men and women.

At high altitude significant increases in oxygen uptakes were observed over sea level controls during four sub-maximal work levels of load carrying and grade walking on the treadmill. Under these conditions, it appears that the energy requirements may be increased at high altitude.

In three studies of caloric restriction for 10 days (complete starvation, 420 Calories of carbohydrate and 500 Cal/day of a protein-carbohydrate diet), it was observed that sub-maximal and maximal work performance on the treadmill was practically unchanged.

BODY OF REPORT

WORK UNIT NO.

Work Performance and Body Composition as Related to Environment and Nutritional Status

PROBLEM:

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The effects of various stresses upon the ability of inilitary personnel to perform their duties are being evaluated. Studies have been done under conditions of abrupt exposure to high altitudes, during ten days of starvation and three studies of caloric restriction, and also on the effect of age in relation to man's ability to perform various military tasks. Investigations of submaximal and maximal work performance and body composition were performed on virtually all studies alluded to under Work Unit Numbers 073 (Applied Nutrition Studies of Military Populations) and 070 and 080 (High Altitude Bioenergetics). Data will be punched on paper tape and an attempt will be made to relate and correlate the parameters that may influence performance (i.e., body fat, body protein, etc.).

-RESULTS AND DISCUSSION OF THE RESULTS:

061

Military Nutrition Surveys: Computer analysis of work performance and body composition data, among other parameters, is well underway for the nutrition surveys at Fort Carson, Colorado; Fort Huachuca, Arizona, and Fort Campbell, Kentucky. This information includes means, standard deviations and correlation coefficients. The data should be available for reporting toward the end of this year.

<u>Caloric Restriction</u>: Data dealing with work performance and body composition of men consuming 420 and 500 Cal/day diets are presently being prepared for publication. Results have shown that during a 10 day period of caloric restriction, both submaximal and maximal work performance on a treadmill, as well as BMR, remained unaltered as compared to control values. It is anticipated that manuscripts on work performance will be forwarded for publication in the immediate future depicting the findings of the 420 and 500 Cal/day studies.

The 1968 Panama study on caloric restriction included measurements of both work performance and body composition. These data are currently being compiled and evaluated. Work Performance and Body Composition as Related to Environment and Nutritional Status (Con't)

High Altitude: These studies have aimed at determining oxygen uptakes in human subjects, abruptly exposed to altitude from sea level (Fort Sam Houston), during 4 and 8% grade walking on a treadmill with and without a 20 kg pack. Results have shown that oxygen uptakes are increased significantly at altitude during work performance at all four levels of activity, compared with control values at sea level. These increases indicate that the energy requirements may be increased at altitude.

Laboratory Procedures: Work is in progress on a duplicate continuous analyzer for measuring oxygen uptake (see USAMRNL Laboratory Report #318, May 1968). This analyzer records continuously on a multipoint, analog strip chart the following parameters of human subjects at rest and during exercise: O₂ and CO₂ concentration, temperature and barometric pressure of expired air; cummulative ventilation, respiration and pulse rate, environmental temperature and relative humidity. Body temperature recording is now being incorporated into the system. The analyzers will be used in all future studies on work performance.

High Carbohydrate Diets: A ten week study has been completed on human subjects at the Metabolic Ward, USAMRNL, to evaluate the effects of high carbohydrate diets on physical work performance. Recent investigations suggest that high carbohydrate diets improve performance. This study will also attempt to delineate the mechanisms by which high carbohydrate diets exert an influence on work performance.

CONCLUSIONS:

The relationship between maximal work performance (oxygen uptake in liters/minute and ml/kg/min), age, respiratory function, and body composition was studied during three nutrition surveys at Fort Carson, Colorado; Fort Huachuca, Arizona, and Fort Campbell, Kentucky. All the data has been punched on paper tape and the means, standard deviations, and correlation coefficients have been calculated. These correlations are now being evaluated for future publications.

In three studies on caloric restriction (starvation, 420 Cal/day, and 500 Cal/day for 10 days) submaximal and maximal work performance was observed to be practically unchanged. Work performance data for the caloric restriction study in the jungles of Panama is now being evaluated.

Work Performance and Body Composition as Related to Environment and Nutritional Status (Con't)

Four levels of submaximal work on the treadmill were evaluated at sea level and high altitude. In all instances, there was an increase in oxygen uptake at altitude indicating that the energy requirements may be increased at high altitude.

RECOMMENDATIONS:

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Other areas to be investigated at high altitude include:

- 1. Mechanisms for the increase in energy expenditure during moderate to heavy physical activity at high altitude.
- 2. Measurement of heavy military tasks at high altitude.
- 3. Continuation of maximal work performance as related to age during nutrition surveys.
- 4. Effects of high protein diets on physical work performance.
- 5. Continuation of studies at altitude and simulated combat to investigate changes in work performance under environmental conditions varying from extreme heat to extreme cold weather.

PUBLICATIONS:

1.

Consolazio, C.F., L.O. Matoush, H.L. Johnson, H.J. Krzywicki, T.A. Daws, and G.J. Isaac. Effects of high carbohydrate diets on performance and clinical symptomatology after rapid ascent to high altitude. Fed. Proc. 28: 937-943, 1969.

- Nelson, R.A., L.O. Matoush, and C.F. Consolazio.
 Development and application of a continuous oxygen uptake measurement system. USAMRNL Report #318, May 1968.
- Consolazio, C.F., H.J. Krzywicki, and R.A. Nelson. Nutritional status in relation to work performance, body composition and age. Proc. 7th Int. Congress of Nutrition, 4: 1-7, 1968.

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ABSTRACT

PROJECT NO.

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TASK NO.

WORK UNIT NO.

Research in Bic-Medical Sciences

Internal Medicine

Muscle Metabolism as Related to Exercise, Serum Electrolytes, Diet, the Influence of Steroids in Normal Man and Disease

STUDY NO. 1: Object: To study the effect of quinine on muscle cation-sensitive membrane ATPase in normal individuals and patients with varying myotonic muscle disease.

Abstract. Myotonia is a state in which muscle relaxation is abnormal. If the removal of calcium from actomyosin is abnormal in myotonic states and if cation-sensitive membrane ATPase is related to calcium movement into the calcium reservoir in the muscle membrane or in muscle sarcoplasmic reticulum then myotonia may be related to an abnormal cation-sensitive membrane ATPase in skeletal muscle.

Normal skeletal muscle obtained by needle biopsy from male volunteers demonstrated that ATPase activity was associated with a 3000 x g fraction. Addition of cations (Ca^{+*}, K⁺, Na⁺, Mg⁺⁺) showed that there was a catiop-sensitive membrane-ATPase in this fraction. Administration of quinine, 300 mg, 3 times a day for a week, to normal volunteers resulted in a statistically significant increase in the cation-sensitive membrane-ATPase. There was no change in the ATPase without the addition of cations.

Patients with myotonia congenita, myotonia dystrophica and muscular dystrophy were found to have low values for this 3000 x g membrane-ATPase. Stimulation with cations was very poor in most of these patients. Administration of quinine led to improvement in one of the patients with myotonia congenita and in one of the patients with myotonia dystrophica. Both of these patients showed marked increases in the cation-sensitive membrane-ATPase in the muscle obtained while they were on quinine therapy. Individuals who did not respond to quinine had little or no change in the cation-sensitive membrane-ATPase of the muscle during quinine therapy. Muscle Metabolism as Related to Exercise, Serum Electrolytes, Diet, the Influence of Steroids in Normal Man and Disease (cont'd)

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Jejunum obtained from normal individuals while on quinine showed a little increase in cation-sensitive membrane-ATL are but not to the degree seen in striated muscle. We conclude from these results that the cation-sensitive membrane-ATPase of normal muscle is sensitive to quinine, and that those patients who respond clinically to quinine have a chemical response to the cation-sensitive membrane-ATPase as well. Patients not responding to quinine do not show the chemical stimulation of this ATPase. Our hypothesis of an abnormal membrane ATPase being involved in myotonic disorders appears valid in some patients.

BODY OF REPORT

WORK UNIT NO. 062

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Muscle Metabolism as Related to Exercise, Serum Electrolytes, Diet, The Influence of Steroids in Normal Man and Disease.

To study the effect of quinine on muscle cationsensitive membrane ATPase in normal individuals and patients with varying myotomic muscle disease,

PROBLEM:

STUDY NO. 1

The relaxation of muscle seems to be related to the removal of calcium from actomyosin. The calcium leaves the actomyosin and is sequestered in a calcium reservoir located in the endoplasmic reticulum (sarcoplasmic Feticulum) of the muscle cell. It has been postulated that cation movement across membranes is related to a cation-sensitive membrane-ATPase located in the membrane. If the relaxation of muscle is related to the removal of calcium and if the calcium moves into its reservoir in the endoplasmic reticulum by virtue of the action of a membranebound cation-sensitive ATPase then any abnormality of this calcium movement due to an abnormal ATPase should result in myotonia. Thus, it might be possible to relate myotonic states to a delect in a muscle membrane cation-sensitive A Pase. If this is true, then those patients who respond to quining with alleviation of their myotonia should demonstrate a change in the muscle cation-sensitive membrane-ATPase as well. In accord with this hypothesis then, normal individuals and patients with myotonia congenita, myotonia dystrophica or muscular dystrophy were studied with and without quinine therapy to determine the activity of muscle cation-sensitive membrane-ATPase of n uscle tissue. Six normal subjects, two patients with myotonia congenita, four with muscular dystrophy and one with adult-onset muscular dystrophy were studied. In addition, jejunal tissue was obtained from the six normal subjects, from one individual with myotonia congenita and from one patient with myotonia dystrophica. The muscle was obtained by percutaneous needle biopsy of the lateral aspect of the thigh. The muscle tissue and the jejunal mucosa were homogenized in buffer and the membranes separated at 3000 x g after removing the

Muscle Metabolism as Related to Exercise, Serum Electrolytes, Dict. The Influence of Steroids in Normal Man and Disease (cont'd)

600 x g sediment. The cation-sensitive membrane-ATPase was assayed with various calions or cation combinations including Mc⁺⁺ Ca^{**}, Mg^{**} + Ca^{**}, Na^{*} + K^{*} and Na^{*} + K^{*} + Mg^{**} + Ca^{**}, ATPase activity was measured by the phosphate (P) liberated in prodes hear has of protein,

RESULTS AND DISCUSSION OF THE RESULTS:

In the normal subjects there was a muscle membrane-ATPase with a mean activity of 5, 15 + 0, 54 µmoles P split/hour mg of protein. When cations were added, for example calcium, a value of 11.0 - 0.96 µmoles P split hour/mg of protein was obtained. When the normal subjects were given quinine for 1 week, 300 mg 3 x day, the value for the muscle membrane ATPase with no cations was 5, 16 = 0, 54. With cations added, for example calcium, a value of $20.6 - 2.1 \,\mu$ moles P split hour mg of protein was obtained. This indicated that a membrane ATPase was present in this fraction, that it was responsive to cations including calcium and that the cation-sensitive activity was markedly increased by quinine administration. The patients all showed decreased membrane-ATPase activity and very poor responses to all of the cations.

After guinine administration one of the patients with myotonia congenita and one of the patients with myotonia dystrophica had a marked clinical improvement in their myotonia with coincident increase in the cationsensitive membrane-ATPase, Another of the patients with involunia dystrophica had a partial improvement and some change in the cationsensitive membrane ATPase. The remaining patients had no or liftle clinical response and no significant change in the cation-sensitive membrane-ATPase activity. There was some change in the jejunal cation-sensitive membrane-ATPase but this was small and did not change to the degree seen in muscle,

CONCLUSIONS:

From these studies we conclude the following:

There is a cation-sensitive muscle membrane-ATPase or ATPases. 1

2. In normal individuals guining potentiates the activity of the cationsensitive muscle membrane-ATPase or ATPases.

Muscle Metabolism as Related to Exercise, Serum Electrolytes, Diet, the Influence of Steroids in Normal Man and Disease (cont'd)

3. Those individuals with myotonia who improve with quining therapy also show responses of the cation-sensitive membrane ATPases.

4. Patients with myotonia congenita, myotonia dystrophica and muscular dystrophy who have a very poor response to quinine therapy have very low activity of muscle membrane-ATPase which responds poorly to the addition of cations.

5. Jejunal tissue has a membrane ATPase which responds to cations but responds poorly to quinine,

HECOMMENDATIONS:

A 1 ST P. S. Mark M. Akada Maharata

The nature of this membrane ATPase should be investigated further. The effect of quinine in vitro should be studied. The relationship between myotonia and dystrophy should be investigated further. This appears to be a very fruitful line of research and will be pursued further.

PUBLICATIONS:

1. R.H. Herman, N.J. DiBella, F.B. Stifel, L. Hagler and N.S. Rosensweig. The effect of quinine on human skeletal muscle and jejunal cation-sensitive membrane-ATPase in normal subjects and patients with myotonia congenita, myotonia dystrophica and muscular dystrophy. Clin. Res. 17: 385, 1969 (Abstract).

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ABSTRACT

PROJECT NO.	3A061102871R	Research in Biomedical Sciences
FASK NO.	02	Internal Medicine
WORK UNIT NO.	063	Studies in Microbial Metabolism

The following investigations have been conducted under this work unit:

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STUDY NO. 1:	Enzymes in M. tuberculosis
STUDY NO. 2:	Carbon metabolism in <u>M.</u> tuberculosis
STUDY NO. 3:	Pathways of amino acid synthesis by M. tuberculosis

1. Due to the transfer of assigned personnel progress in this area has been reduced. Earlier investigations which established differences in the NADH-diaphorase content of the isoniazid-resistant M. tuberculosis as compared to the drug-susceptible strain have been reported in two journal publications. In new studies an attempt is being made to relate the difference in diaphorase content of the isoniazid-resistant organism to change in the respiratory pathway normally present in M. tuberculosis. Using an oxygen analyzer it has been possible to show the uptake of oxygen by cell-free extracts of M. tuberculosis and the susceptibility of this uptake to inhibition by classical respiratory inhibitors.

2. Completed studies which described the utilization of D-glucose-¹⁴C by <u>M. tuberculosis</u> and strains resistant to INH, PAS, and streptomycin have been submitted for journal publication. Based on the finding that large amounts of D-glucose is converted to trehalose, investigations concerned with the significance of this compound in the nutrition and pathogenicity of <u>M. tuberculosis</u> are currently in progress.

3. Using the technique of "isotopic competition" biosynthetic relationships between groups of amino acids have continued to receive attention. It has been possible to demonstrate in M. tuberculosis that certain key intermediates, such as pyruvate, L-aspartic, and L-glutamic, furnish the main carbon skeleton for groups of amino acids. Although pathways of amino acid synthesis show similarity with other microorganisms, M. tuberculosis has exhibited considerable versatility in the utilization of carbon compounds and was found to employ unexpected pathways in the synthesis of certain amino acids.

BODY OF REPORT

WORK UNIT NO. 063

Studies in Microbial Metabolism

STUDY NO. 1

Enzymes in M. tuberculosis

PROBLEM:

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To compare the enzyme content of drug-susceptible and drug-resistant Mycobacterium tuberculosis.

RESULTS AND DISCUSSION OF RESULTS:

Previous studies in our loboratory have shown that isoniazid-resistant M. tuberculosis possesses more NADH-diophorase activity than the drug-susceptible strain. To establish if this apparent metabolic difference between the two organisms is meaningful in terms of the emergence and survival of the isoniazid-resistant organism has been the goal of current investigations. Speculation that this finding reflects change in the normal respiratory pathway is strengthened by the fact that the INH-resistont organism has olso lost catalase. Of possible significance to the present case is the work of Brodie, who showed that diaphoraselike enzymes may bypass segments of the normal respiratory chain, reentering later at the cytochrome c level. To dote, efforts have been concerned with the establishment of a suitable test system. Employing on oxygen analyzer, it has been possible to show oxygen uptake with cell free extracts of M. tuberculosis. This uptake was found to be associated with a particulate fraction obtained by ultrocentrifugation. NADH was furnished in substrate quantity or formed through a NADH generating system of ethonol-ethanol dehydrogenase or B-hydroxy-butyric acid-B-hydroxy-butyric acid dehydrogenose. Respiration was inhibited by amytal, quinoline N-oxide, p-chloromercuribenzoate, and potassium cyonide. The foilure to demonstrate oxidative phosphorylation in these preparations may reflect the high endogenous ATPase activity.

CONCLUSIONS:

According to Brodie the respiratory pathwoy in <u>Mycobacterium</u> phlei may be depicted in the following manner:



By inhibiting respiration with quincline N-oxide and amytol the extent of "electron bypass" occurring in isoniazid-susceptible and isoniazid-resistant M. tuberculosis can be evaluated.

RECOMMENDATIONS:

It is proposed that this work be continued with the availability of personnel.

PUBLICATIONS:

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- 1. O'Barr, T. P., and Smith, M. A. Comparative NADH-diaphorase content of isoniazid-resistant and isoniazid-susceptible Mycobacterium tuberculosis. Amer. Rev. Resp. Disease, 99:116, 1969.
- O'Barr, T. P. Rapid electrophoretic examination of diaphorases in Mycobacterium tuberculosis. Proc. Soc. Exptl. Biol. Med., 131, 1969.

STUDY NO. 2

Carbohydrate Metabolism in M. tuberculosis

PROBLEM:

To obtain information concerning carbohydrate metabolism in drug-susceptible and drug-resistant Mycobacterium tuberculosis.

RESULTS AND DISCUSSION OF RESULTS:

Following the demonstration in our laboratory that considerable amounts of D-glucose is converted to trehalose by M. tuberculosis, the nutritional and physiological significance of this compound has been of interest. A variety of evidence points to a unique role for trehalose in the growth and pathogenicity of M. tuberculosis. Trehalose-6, 6-dimycolate (cord factor) is toxic for laboratory animals, causing loss of weight and death in mice. It has also been proposed that the acquired resistance to tuberculous infectior. achieved by immunization with cell wall preparations is due to the presence of a cordfactor like component. From a nutritional sense, trehalose may play an important role in the growth of M. tuberculosis, since trehalose-amine has been shown to possess antimycobacterial properties. The approach employed ¹⁴C has been to prepare carbon-14 labeled trehalose and study its incorporation into various cell fractions. Data thus far obtained has shown that trehaloseis readily fixed into lipid, nucleic acid, and residue fractions. It is planned to subject these fractions to more detailed analyses through the use of chromatography and chemical fractionation.

Within this work area a collaborative study concerned with developing methodology for the quantitative trapping and estimation of ${}^{14}CO_2$ has been carried out.

CONCLUSIONS:

Using carbon-14 labeled trehalose it has been possible to show incorporation of radioactivity into cell fractions of M. tuberculosis.

RECOMMENDATIONS:

It is recommended that the lipid fraction extracted from <u>M</u>. tuberculosis after incubation with trehalose be examined for trehalose-lipid components which might possess toxic as immunogenic properties.

PUBLICATIONS:

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1. O'Barr, T. P. and Rothlauf, M. V. Metabolism of D-glucose by <u>Mycobacterium tuberculosis</u>. Submitted for journal publication May 1969.

 Whitten, B. K., Beecher, G., Liddle, C. G., and O'Barr, T. P. A comparison of ¹⁴CO₂ trapping agents used in <u>in vitro</u> metabolic studies. USAMRNL Laboratory Report in preparation.

STUDY NO. 3

Pathways of Amino Acid Synthesis by M. tuberculosis

PROBLEM:

To examine the pathwoys of amino acid synthesis in drug-susceptible and drug-resistant Mycobacterium tuberculosis.

RESULTS AND DISCUSSION OF RESULTS:

Employing experimental procedures described in the 1968 Annual Research Progress Report, studies concerned with pathways of amino acid synthesis in M. tuberculosis have continued. Briefly, in the "isotopic competition" M. tuberculosis have continued. Divery, in the technique a control pattern of radioactivity in protein-bound amino acids is 14 Local modia containing D-alucose - C established for M. tuberculosis grown in basol media containing D-glucoseas the sole source of carbon. The control pattern furnishes a basis for comparison with labeling observed in protein-bound amino acids when M. tubercutosis is grown in D-glucose-¹⁴C media supplemented with nonradioactive amino acids. Not only will radioactivity be suppressed in the supplemented amino acid but also in amino acids that are related through biochemical pathways. In this manner families of amino acids have been established in the metabolism of M. tuberculosis, Aspartic acid suppresses octivity in a large number of amino acids but more so in isoleucine, threasine, methionine, and lysine. Glutamic finds its way into a number of amino acids but is converted directly to arginine and proline. Pyruvate is incorporated into valine, leucine, olanine, and serine and glycine. Glycine and serine are readily interconvertable. An unexpected finding was the significant displacement of radioactivity in serine by homoserine. The supplementation of basal media with amino acids revealed several examples of growth suppression and inhibition. L-homoserine effectively prevented growth from standard inocula of M. tuberculosis. The site of inhibition is not known but arowth of L-homoserine-inhibited cells is restored by supplements of L-valine and L-leucine. The complex nature of the relationship is revealed by the fact that in the absence of L-homoserine, valine and leucine are inhibitory. In other examples, the addition of L-homocysteine to actively growing cultures caused an immediate cessation of growth, and a-ominobutyric wos found to inhibit growth. Consideration is being given to the possibility that these antagonisms represent examples of "feed back" inhibition or "suppression". It is interesting to speculate that such complex relationships have a bearing on the slow growth of M. tuberculosis.

CONCLUSIONS:

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3. Data to date indicate that many amino acids are synthesized in M_i tuberculosis by pathways that are similar to those found in other microorganisms, although differences have been noted which may have unique significance to the metabolism of M_i tuberculosis. Certain intermediates in the synthesis of amino acids were shown to exert control over the growth of M_i tuberculosis.

RECOMMENDATIONS:

Continue to study pathways of synthesis and nutritional interrelationships of amino acids in the metabolism of M. tuberculosis.

PUBLICATIONS:

Manuscript reporting pathways of amino acid synthesis by <u>M. tuberculosis</u> is in preparation.

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ABSTRACT

PROJECT 30. 3A0011028718

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TASK DO. UL

SORK UNIT NO.

Internal Hedicine

Biomedical Information Systems Design

Research in Blomedical Sciences

The following investigations have been conducted under this work unit:

STUDY NO. 11 The Evaluation of Long-Term Use of Otal Contraceptives

STUDY NO. 21 The Development of a Lottral (Loglion) Language Information System for the Tumor Research Registry, Fitzsimons General Rospital

STUDY NO. 31 General Programming and Support Development

This work unit serves to develop methods for automating medical data in cooncration with Fitzsimons General Hospital. Coordination with the hospital is through the services concerned and Clinical Research Service.

Study no. 1, with the OB-GYN Service. is a system to collect, slute, rotrieve and analyze patient data relevant to the evaluation of oral contraceptives.

Study no. 2, with the Tumor Registry, is the development of a computer based system which will accept the English prose generated by the physician and maintain this information in a logical file system. The people involved are not required to generate any coding material.

Study no. 3, serves to develop the necessary supporting program for the effective use of the computer. This work includes the control system programming, language usage programming, some statistical programming and support.

BODY OF REPORT

MORK UATT NO. 064

Biomedical Information Systems Design

STUDY NO. 1

The Lvaluation of the Long-Term Use of Oral Contraceptives

PROBLEM

There is a need to develop computer based information systems which have the capability to process the requirements for evaluating long term medical regimens. Such programs present unique problems as regards information processes of data collection, storage, analysis and retrieval. The long-term administration of oral contraceptives provides an excellent model for such an information system. Additionally, it is necessary to continuously monitor and evaluate the effects of long-term use of oral contraceptives through statistical methods and systematic classification techniques.

RESULTS AND DISCUSSION OF THE RESULTS:

The study was begun on 1 March 1967. A questionnaire was administered to each patient upon entry into the OB-GYN family planning section. A physician summary was completed for each of the patients. A number of subjects were selected from other sections of the service to provide additional information. This data was continuously updated to form a chronological patient record which could be accessed under physician command. A retrieval system was developed to produce a report to the physicians based on individual or group analysis. Information generated by this report showed that the data being collected did not give the criteria to make an evaluation of the effects of the oral contraceptives. The data collection procedure was revised to include more physician generated information (checklists and summaries) and data concerning other drugs which the subjects could be taking. Provisions have been made to include physicians' comments as a part of the subject record. The new data formats are being stored in the patient record and a new system of interrogation is being developed.

CONCLUSIONS!

The new data collection provides a method consistent with the examination procedures used in the OH-GYN Service. The data being collected is more relevant to the evaluation of the effects of the oral contraceptives.

Statistical routines have been developed which can perform the necessary data manipulation necessary for evaluation of the information.

Biomedical Information Systems Design (Cont'd)

RECOMMENDATIONS:

1. Development of program to facilitate information storage should be continued.

2. Establish mattern recognition programs for feedback to the examining physician.

STUDY NO. ?

The Development of a Haturel (English) Language Retrieval System for the Tumor Research Registry, Fitzsimons General Hospital

PROBLEM:

Methods tailored to the structural and functional characteristics of the electronic digital computer must be developed to process, under automatic controls, medical information documented in natural language. Past experience has demonstrated that the time-honored technique of assigning codes to information documented in natural language prosrives rise to formidable problems. This study aims to achieve a means for processing the computer input and retrieval of information in the English Language familiar to medical personnel. These functions must be achieved with no encoding of information prior to computer input or preliminary to communication with the computer, and no decoding of information output from the computer.

RESULTS AND DISCUSSION OF THE RESULTS:

A programming system to take unformated and uncoded Tumor Registry data is being written to facilitate the information handling of large bodies of unstructured data. This program will convert the data into a structured file format for update in the general patient master file. This data will be stored in a retrievable form under conditions of logic expressed by the participating service. The data may be retrieved by location, disease, process, modifier processes, person, time or any combination of the preceeding. Follow-up reports will be generated by programming system.

CONCLUSIONS:

Based on the beginning programming effort, it was discovered that a concept of fixed input formats only did not resolve the problems of information handling. The formats did not allow the latitude needed for discussion of complicated cases which occur. Biomedical Information Systems Design (Cont'd)

The solution has been to augment usable formats with non-formatten data for a full coverage of the case involved.

RECONCLUDATIONS:

Continue the program development needed for the implementation of the system.

STUDY NO. 3

General Programming and uncort. Development

PROBLEM:

To facilitate the application of the computer, certain basic programming must be developed. This type of programming is of a general nature and applicable to many different projects.

RESULTS AND DISCUSSION OF RESULTS:

Two programming languages, 301 Assembly and Fortran II, were implemented as standards by the Computer Division. These replace the 301 Machine Language as the standard. The Assembly Language is used for all programming except that of a mathematical or statistical nature. The Fortran II is used for the mathematical and statistical programs.

A Program Library Tape system has been instituted for the loading and execution of the routines used. This tape contains a resident control program which remains in core memory throughout program execution and the programs developed by the division.

A Pseudo Code Tape is now maintained for all programs written. This tape contains the source program which can be modified and reassembled if the need arises.

The programs which are applicable to all of the projects are being developed. These are: General Data File, General System File, Mathematical, and Statistical routines. Some data collected by other divisions of the laboratory and the Clinical Research Service has been analyzed through these general programs. The following support was provided:

1. Psychology Branch - statistical analysis and equipment

- consultation
- 2. Physiology statistical analysis
- 3. Chemistry statistical analysis

Biomedical Information Systems Design (Cont'd)

4. Metabolic - mathematical and statistical consultation

- Cardiology statistical analysis
 Pulmonary Function statistical and mathematical analysis and consultation

RECOMMENDATIONS:

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1. Development of general routines be continued.

2. The statistical and mathematical support be done under a new protocol.

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ABSTRACT

PROJECT NO.

3A061102B71R

Military Environmental Medicine

WORK UNIT NO. 080

High Altitude Bioenergetics (Basic)

Feeding high carbohydrate - low fat diets reduced the clinical symptoms observed after abrupt altitude exposure and increased maximal work capacities, so two more human studies were performed.

In the first study, 16 men were fed 1800 calories of normal foods. Eight of these men (6 exercisers, 2 sedentary) received 1800 calories of carbohydrate - protein liquid; the remaining 8 (5 exercisers, 3 sedentary) received 1800 calories of carbohydrate-fat-protein supplements. Caloric consumption averaged over 3300 Cal/day at altitude (35-3600 at sea level) in all except the normal supplemented sedentary subjects. Oral glucose tolerance tests were normal, nitrogen balances were positive and no body weight or water losses were found at altitude which is in contrast to observations from previous studies where food consumption was greatly reduced after abrupt altitude exposure. It would appear that many of the effects previously attributed to altitude exposure were due to caloric restriction per se.

The second study was performed on 6 conscientious objector volunteers from the Metabolic Ward. Three of these men received a high carbohydrate-low fat liquid diet, and 3 received the normal caloric distribution diets. The men were studied for 9 weeks during physical training at Denver, and then the measurements were repeated during 3 days at altitude. Measurements of intravenous glucose tolerances, water balances, calories, nitrogen, and minerals, physical performance, body composition, and changes of free fatty acids, triglycerides and cholesterol in blood during treadmill walks were performed, but the analyses of samples and data have not been done.

BODY OF REPORT

WORK UNIT:

080

High Altitude Bioenergetics (Basic)

PROBLEM:

The detrimental effects of abrupt altitude exposure upon physical performance, physical well-being, and mental functions have been studied along with the influence of high carbohydrate - low fat diets upon these effects. Since these diets were beneficial in reducing the altitude syndrome, the intermediary metabolism of energy and nitrogen could yield further information on the mechanisms involved in causing the nausea, headaches, etc., and those which are affected by diets. This information could indicate means of enhancing the beneficial effects of the diets by selective use of carbohydrate. To obtain this information, two studies have been performed with human volunteers and further studies have been planned in both man and animals.

RESULTS AND DISCUSSION OF RESULTS:

In the 1968 study, 8 men (6 exercisers, 2 sedentary) received a high carbohydrate - low fat diet, and 8 men (5 exercisers and 3 sedentary) a normal diet. All of the men received 1800 calories of normal foods and 1800 calories of either high carbohydrate liquid or normal caloric distribution liquid diets. The men consumed 3300 calories per day at altitude, and oral glucose tolerance tests were not affected by altitude. This is in contrast to other studies when the subjects did not consume more than about 800-1200 Cal/day. With adequate caloric intakes, the previously observed body weight, water and nitrogen or protein losses were not found. This would indicate that many of the effects previously attributed to altitude exposure or hypoxia were due to the reduction of caloric consumption at altitude. High carbohydrate - low fat and/or moderate to heavy exercise reduced the severity and duration of clinical symptoms after abrupt altitude exposure.

In the 1969 study (completed June 1969), 6 men (3 receiving a normal composition liquid diet, and 3 a high carbohydrate - low fat diet) were studied extensively at Denver while training to determine the effects of diet at this elevation. These studies included complete balances of water, calories, nitrogen and minerals (Na, K, Ca and Mg), maximal
High Altitude Bioenergetics (Basic)

work performance, oxygen consumption, body compositional changes, intravenous glucose tolerance tests, and changes in blood levels of free fatty acids, triglyceride and cholesterol during rest and exercise. All of the tests were repeated at biweekly intervals and once at altitude. Analyses of the samples and data have just begun, so very few results are known. From work times on the maximal performance tests, the differences in diets did not appear to affect physical performance at the Denver altitude.

CONCLUSIONS:

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By feeding one-half normal foods and one-half of the calories as liquid diets, caloric consumption was maintained during abrupt altitude exposure with the prevention of decreased glucose tolerances, hypohydration, body weight losses and negative nitrogen balances usually associated with early exposure to altitude. Symptomology was greatly reduced by both high carbohydrate low fat diets and physical activity.

Since the 1969 altitude study has just been completed, analyses of samples and data have not been performed so that no conclusions are available.

RECOMMENDATIONS:

Completion of work started on the 1969 altitude study and preparation of reports of the results and conclusions.

Further studies on the metabolism of glucose at altitude using 14-C glucose to ascertain pool sizes, hepatic release of glucose from glycogen stores and glucose uptake and utilization by peripheral tissues.

Animal studies on the influences of diet and exercise upon various enzymic activities involved in energy utilization and storage and in protein anabolism and catabolism. 脑脑筋脑 的复数 网络人名里兰米

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pulmonary function. The basic aspects of cardiopulmonary physiology will be emphasized including the chagges in hemodynamics, regional blood flow, blood composition, myocardial function, etc. The relationships and/or influence of the cardiopulmonary function on other body systems or functions will also be investigated.

24. (U) Approach: These studies will be conducted primarily in laboratory animals. but on occasion, wild species, e.g., thirteen-line ground squirrels, will be employed. Generally, standard physiological and biochemical procedures will be used, depending upon the particular functional aspect which is to be investigated. The studies will be conducted under various hypoxic gas mixtures, in environmental test chambers, or at actual high altitude field sites. The latter will be used primarily for long-term or acclimatization studies.

25. (U) Progress (Jul 684Jun 69) Preliminary experiments have demonstrated that high altitude exposure increases cerebral spinal fluid pressure and common carctid blood flow. Rat brain water (dry weight/wet weight) was not altered after four hours' exposure to 20,000 feet altitude, by the administration of 5 mg/kg furosemide. There was no statistical difference between brain water content of low and high altitude exposed animals. Further studies will be continued under work: unit 082 Metabolic Effects of Altitude, Agency Accession DA OA 6339.

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ABSTRACT

PROJECT NO.	3A 061102B71R	Research in Biomedical Sciences
TASK NO.	05	Environmental Medicine
WORK UNIT NO.	081	Cardiovascular and Pulmonary Responses at High Altitude

The following investigation has been conducted under this work unit:

STUDY NO. 6: The Response of the Coronary Vasculature and Tissue Fluid Compartments to Decreased Oxygen Supply

Recent studies of acute mountain sickness (AMS) have reimplicated cerebral edema and cerebrospinal fluid hypertension in its etiology. As a result, increased cerebral blood flow has been suggested as a determinant of the edema and hypertension.

Unanesthetized dogs implanted with carotid artery electromagnetic flowmeters and cisternum magnum cannula, connected to a pressure transducer, were studied at 5,280 ft. and during a slow ascent (30 minutes) to 14,000 ft. in a hypobaric chamber. During the ascent, total carotid flow increased. Cerebrospinal fluid pressure rose immediately to 100 mm H_2O when the ascent began. It then slowly rose to 450 mm H_2O during the ascent. During descent, it returned to control values within 30 minutes after the chamber was reduced to ambient pressure.

Male Holtzman rats (300 g) were given 20 mg Furosemide/kg orally. Six control and six Furosemide treated animals were rapidly brought to 20,000 ft. in a hypobaric chamber. They remained there for four hours. After rapid descent they were sacrificed, brains were sampled, weighed and dried at 50° C to constant weight. No difference was observed between the dry/wet tissue ratio of both groups of animals. Moreover, no statistical difference was observed between this ratio in low and high altitude exposed animals.

The results of this study have suggested that at least some of the symptoms of AMS are related to increased cerebral blood flow and cerebrospinal fluid hypertension but not to cerebral edema.

BODY OF REPORT

WORK UNIT NO. 081

STUDY NO. 6

Cardiovascular and Pulmonary Responses at High Altitude

The Response of the Coronary Vasculature and Tissue Fluid Compartments to Decreased Oxygen Supply

PROBLEM:

Acute mountain sickness (AMS) is a syndrome that occurs in a significant part of sea level dwellers who are transported to high altitude (14,000 ft.) and remain there. Studies have implicated a redistribution of blood volume such that cerebral blood flow increases causing cerebral edema and/or cerebrospinal fluid hypertension. The severity of this illness reaches a peak during the first two days at altitude and decreases to minimal ievels after 4 to 7 days at altitude. This study is directed toward relating the course of AMS with changes in cerebrai blood flow, cerebrospinal fluid pressure and cerebral water.

RESULTS AND DISCUSSION OF RESULTS:

Unanesthetized dogs implanted with carotid artery electromagnetic blood flow probes and a cannula inserted into the cisternum magnum were used. The cannula was connected to a pressure transducer. The animal was placed in the hypobaric chamber, control measurements were made. The chamber was then evacuated slowly to rise at the rate of 300 ft/min. The pressure in the chamber, therefore, reached a pressure equivalent to 14,000 ft. altitude in 30 minutes. The animals were kept at this pressure for two hours. The chamber was then pressurized at the same rate as it was evacuated. Cerebrospinal fluid pressure (CSFP) was originally 10 mm H2O; as the evacuation procedure started, it rose immediately to 100 mm H2O; during the evacuation it slowly rose and continued to rise during the 2 hour period at 14,000 ft, pressure to 450 mm H2O. At the start of pressurization, CSFP dropped 100 mm H₂O immediately; it slowly declined during pressurization and reached control levels within 30 minutes after ambient pressure was achieved. During the experiment, carotid blood flow was significantly increased above control. However, the flow increased immediately upon evacuation and remained at this level through the periods of evacuation, maintenance and pressurization. It returned toward control during the period after the chamber reached ambient pressure.

Cardiovascular and Pulmonary Responses at High Altitude (Cont'd)

Brain water (dry/wet tissue) of rats kept at 5,280 ft. was not different from brain water of rats exposed to a pressure equivalent to 20,000 ft. altitude: 80.1% vs 79.3%. Six rats were given Furosemide 20 mg/ kg orally, placed in the hypobaric chamber and evacuated to the 20,000 ft. equivalent pressure. After four hours at this pressure, the animals were rapidly brought to ambient pressure. The dry/wet brain tissue ratio of these animals was 81.4%. In this method of determining brain water content with and without a diuretic, altitude was not effective in altering brain water. However, the dog experiment did show that altitude causes cerebrospinal fluid hypertension.

RECOMMENDATIONSI

1. Further studies are needed to ascertain changes in cerebral blood flow, cerebrospinal fluid pressure and production, and their interrelation during acute high altitude exposure.

2. Due to the loss of two civilian scientists because of manpower reductions imposed upon this Laboratory, to a lesser extent, the reduction in financial support and to effect better management of resources, research under this work unit will be discontinued. Future efforts of this work unit will be transferred in FY 70 to Work Unit 082, Metabolic, Physiological and Psychological Effects of Altitude.

PUBLICATIONS

None

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ABSTRACT

PROJECT NO.	3A061102B71R	Research in Biomedical Sciences
TASK NO.	05	Environmental Medicine
WORK UNIT NO.	082	Metabolic Effect of Altitude

The following investigations have been conducted under this work unit:

STUDY NO. 8: Comparative Lipid Profiles of Serum from Rats, Dogs and Humans Exposed to High Altitude (14, 100 ft.)

STUDY NO. 9: Effects of Aititude on the Myocardium of Animals

STUDY NO. 10: Relative Effects of High Altitude and Concomitant Anorexia on Weight Gain and Body Composition of the Rat

STUDY NO. 11: Effect of Altitude on Metabolic Fecal Nitrogen and Endogenous Urinary Nitrogen

In most species, including man, high altitude exposure is associated with a loss in body weight. Studies in this division have indicated that the weight loss is largely due to a reduction in body fat. Altitude stress also resulted in a decreased efficiency of food utilization. However, the relative significance of hypoxia per se and the hypoxia-induced anorexia on the loss of body weight has not been delineated. The present studies were aimed at an understanding of the change in lipid and nitrogen metabolism during acute high altitude stress. In the serum lipid profile investigation (Study 8) ten humans were studied for 14 days at sea ievel and nine days at high altitude (14, 100 ft). Serum lipid profiles, determined on Day 12 at sea level and on Days 1, 4, 6, 7, 8 and 9 at altitude showed that free fatty acids rose on the first day at altitude and remained there and triglycerides were above control from Day 6 to Day 9 at altitude. These changes probably reflected neuroendocrine aiterations in mobilization of stored lipids during high altitude stress. in Studies 9, 10 and 11 animals were used. Mitochondrial oxidation from myocardial and striated muscle of dogs, rat and rabbit was examined at 5, 250 ft. and after three months at 14, 100 ft. Aititude exposure had little effect on striated muscle. Canine myocardiai mitochondria from exposed animals had an increased ability to oxidize palmitate-i-14C to i4CO2. Altitude exposure had little effect on rat heart palmitate oxidation. There was a decreased ability of rabbit myocardial mitochondria to oxidize palmitate. The observations

Metabolic Effect of Altitude (Cont¹d)

suggest a similarity between the effect of altitude exposure on rabbit and rat myocardium and the effect of artificially-induced heart failure in experimental animals. In Studies 10 and 11, the weight loss of 14, 100 ft. altitude exposed animals was similar to that of 5, 280 ft. pair-fed control animals. Increased rates of endogenous urinary nitrogen and metabolic fecal nitrogen were not observed in altitude exposed animals. Weight loss at altitude seems to be related to anorexia rather than hypoxia.

BODY OF REPORT

WORK UNIT NO. 082 STUDY NO. 8 Metabolic Effects of Altitude

Comparative Lipid Profiles of Serum from Rats, Dogs and Humans Exposed to High Altitude (14, 100 ft.)

PROBLEM:

In most species, including man, acute high altitude exposure is associated with a loss of body weight. Studies in this division have indicated that weight loss is largely due to a reduction in body fat. Several factors may be responsible for the decrease in body fat at high altitudes. Data in the open literature indicate that hypoxia, caloric restriction and malabsorption may all be involved in the loss in body fat at high altitude. Changes in lipid metabolism at high altitude may be reflected by alterations in serum lipids. Only a few studies pertaining to the effect of acute high altitude exposure on serum lipids have been reported. In the present study, serum FFA, phospholipid, triglyceride and cholesterol fractions were measured and the fatty acid composition of these lipids determined in ten healthy male subjects maintained on a liquid diet for 14 days at sea level and during a nine-day exposure to an altitude of 14, 100 feet. Alterations in the total concentration and fatty acid composition of these serum lipid fractions were correlated with changes in caloric intake. The effects of hypoxia and caloric restriction on lipid metabolism at high altitude are discussed.

RESULTS AND DISCUSSION OF RESULTS:

A mean weight loss of 4.27 kg. occurred during the nine-day exposure period at 14, 100 ft. A marked reduction in caloric intake, falling to 250 g, occurred on the first day of altitude exposure. Caloric intake rose to 1500 g on the fifth day of exposure, then declined again. A caloric reduction of this magnitude would probably be accompanied by a more extensive catabolism of tissue than was observed in previous studies where solid diets were used. This factor may account for the greater weight loss reported in this study.

Serum FFA increased on Day 1 at 14, 100 ft. and remained elevated throughout the high altitude exposure. There was a significant negative correlation (r = 0.69, P<0.001) between serum FFA levels and caloric intake. Serum triglycerides were significantly higher on

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Days 6, 7, 8 and 9 while no change in total cholesterol or serum phosphorous was observed.

The rise in serum FFA levels observed at 14,100 ft. may, in part, be explained by an increase in sympathetic activity. Hypoxia, however, is associated with caloric restriction which will also cause an increased serum FFA. In the present study, a significant negative correlation between serum FFA levels and caloric intake was found. This would suggest a direct effect of caloric restriction on serum FFA. It is known that adrenal cortical activity is increased during acute high altitude exposure. Glucocorticoids, growth hormone and thyroxine can change serum lipid levels and FFA concentrations. However, it is known that starvation or semi-starvation will stimulate increases in FFA.

RECOMMENDATIONS:

1. Serum lipid profiles should be determined in humans exposed to high altitude and who maintain their weight closer to sea level values.

2. Serum lipid profiles should be determined in humans fed various diets and exposed to altitude.

3. Free fatty acid turnover should be monitored during exposure to high altitude.

STUDY NO. 9

Effects of Altitude on the Myocardium of Animals

PROBLEM:

Studies in this laboratory during the past two years have shown that rats, rabbits, cats, and dogs exhibit varying degrees of right heart hyperticy vafter prolonged (3 months) exposure to altitude (14, 100 ft). Concomit int with hypertrophy, ultrastructural changes were observed in mitochondria of the ventricular myocardium from dogs, rabbits, cats, and rats. These ultrastructural changes consisted of markedly swollen mitochondria containing irregularly arranged and reduced numbers of cristae. Dogs, rabbits and rats were exposed to 14, 100 feet for three months. Isolated mitochondria from myocardial and striated muscle were studied. The determinations made were: ability to oxidize palmitate-1- 14 C to 14 CO₂, oxygen uptake, glycolysis

and phosphate potential.

RESULTS AND DISCUSSION OF RESULTS:

The above was a collaborative study among the personnel of Physiology, Chemistry and Pathology Divisions, USAMRNL, and USARIEM. A detailed report of the findings is contained under Work Unit 085 "Effects of Altitude on the Myocardium of Animals."

STUDY NO. 10

Relative Effects of High Altitude and Concomitant Anorexia on Weight Gain and Body Composition of the Rat

PROBLEM:

Many investigators have observed decreased growth rates and altered body composition in animals exposed to actual or simulated altitudes greater than 11,000 feet. The exact nature and causes of the growth depression remains unclear. Dehydration, decreased food efficiency, depressed nutrient digestibility and/or absorption, and altitude-induced reduction of voluntary food intake have been suggested to be involved. Most high altitude investigations have been conducted with the diet being offered ad libitum to both experimental and control groups. Diminished appetite and caloric consumption during at least the first week of exposure are frequent observations in both man and animals. Accordingly, it is difficult to distinguish the metabolic effects of high altitude per se from those of the altitude-induced distary deprivation. The objective of this study will be to determine, utilizing paired feeding techniques and a normal diet, the relative significance of the hypoxia per se and the concomitant hypoxia-induced anorexia upon the following metabolic variables in the rat following 2, 7 or 14 days' exposure to 14, 100 feet:

1. Body weight change

- Body composition changes to include total body water, fat, protein and mineral
- 3. Apparent nitrogen digestibility
- 4. Nitrogen balance and retention.
- 5. Efficiency of food utilization

RESULTS AND DISCUSSION OF THE RESULTS:

This study will be conducted during August 1969.

STUDY NO. 11

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Effect of Altitude on Metabolic Fecal Nitrogen and Endogenous Urinary Nitrogen

PROBLEM:

Exposure of animals to alkitudes above 12,000 feet results in depressed growth rate, growth efficiency and body fat deposition. Data previously reported from this laboratory showed that although the altitude animals consumed less protein, they excreted a significantly greater amount of fecal and urinary nitrogen than the low altitude animals. The data indicated an impaired digestion and/or intestinal absorption of nutrients in rats exposed to high altitude or possibly increased endogenous turinary nitrogen (EUN) and metabolic fecal nitrogen (MFN) excretion. Accordingly, the EUN and MFN excretion patterns and body compositional changes were examined in rats exposed to 14, 100 ft.

Forty-eight male Holtzman rats (300 g) were divided into three equal groups; Pikes Peak ad libitum fed. Denver ad libitum fed and Denver pair-fed. The animals were fed a lab chow diet for one week prior to the experimental period and housed in individual metabolism cages. The high altitude animals were then transported to Pikes Peak (14, 100 ft.) and all animals were fed a "protein-free" diet for 14 days. The Denver pair-fed group was fed amounts based upon their individual high altitude pair-mate's voluntary consumption. Daily changes in body weight and food consumption were measured. Individual daily fecal and urine collections were made during the preliminary and experimental periods for total nitrogen determination. All animals were sacrificed for body composition analysis at the conclusion of the study.

RESULTS AND DISCUSSION OF THE RESULTS:

Body weight loss increased and voluntary food intake was depressed at altitude compared to the ad libitum-fed control groups. The weight loss of the pair-fed control group was not different from that of the altitude group, suggesting that the increased weight loss at altitude could be almost solely attributed to the resulting anorexia. Increased rates of EUN and MFN excretion were not observed in rats exposed to

high altitude. Under the conditions of this study, body composition was not altered by high altitude exposure. The chemical and statistical analyses are 80% completed at this time.

CONCLUSIONS:

Preliminary data indicate that the nitrogen excretion rate coming from endogenous sources is not increased during longer term altitude exposure. Paired feeding indicated that the increased weight loss at altitude reflected an altitude-induced anorexia rather than hypoxic exposure per se.

RECOMMENDATIONS:

Continue to compile and evaluate the data for a near future publication. The following studies are recommended utilizing a normal diet:

1. The relative effects of high altitude and concomitant anorexia on weight gain and body composition of the rat.

2. Effects of high altitude exposure and concomitant anorexia upon fat deposition.

Due to the inability to hire replacements for two civilian scientists who transferred from the Unit and could not be replaced due to a hiring freeze, to a lesser extent the reductions in financial support, and to effect better management of resources, research under this work unit will be curtailed. Future applied studies in cardiopulmonary physiology, metabolism and performance at altitude will be consolidated under this Work Unit 082 which will be entitled Metabolic, Physiological and Psychological Effects of Altitude.

PUBLICA TIONS:

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2. Whitten, B.K., J. P. Hannon, G. J. Klain and K. S. K. Chinn. Effect of high altitude (14, 100 ft.) on nitrogenous components of human serum. Metabolism 17:360-365, 1968.

3. Whitten, B.K. and A.H. Janoski. Effects of high altitude and diet on lipid components of human serum. Fed. Proc. 28:983-986, 1969.

4. Klain, G. J. and J. P. Hannon. Effects of High Altitude on Lipid Components of Human Serum. Proc. Soc. Experimental Biol. Med. 129:646-649, 1968.

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ABSTRACT

PROJECT NO.	3A0611021371R	Research in Diomedical Science
TASK NO.	05	Environmental Medicine
WORK UNIT NO.	083	Physiological and Psychological Aspects of Performance at Altitude

Detailed analyses were carried out on the symptomatology of Acute Mountain Sickness (AMS) and mental performance of 23 male subjects (18-23 years old) rapidly transferred from a sea level site to an altitude of 14, 110 feet, With one exception (LIVELY), all symptoms measured by the General High Altitude Questionnaire (GHAO) changed significantly during exposure to high altitude with HEADACHE showing the largest change. Key cluster analyses were performed on the low and high altitude GHAQ data to Identify sets of symptoms which changed together. Three clusters with low interrelationships were identified and given superordinate class names reflecting the essential features of cluster symptomst 1) Arousal Level (ACTIVE, COMFORTABLE, ENERGETIC, REFRESHED, and VIGOROUS), 2) Somatic Discomfort (NAUSEA, SHORT OF BREATH, DIZZY, and HEART POUNDING), and 3) Mood (HAPPY, SATISFIED, PLEASED), Both Arousal Level and Somatic Discomfort were found as a high altitude cluster, while Mood was a low altitude cluster,

Mental performance generally decremented at high altitude for those subjects reporting the most severe symptoms on the GHAQ. Through multiple regression analyses, the performance decrement on the mental tasks was found to be highly related to the severity of individual symptoms of AMS. The performance decrement in tasks requiring motor activity (e.g., writing, reaction time) was related to the symptoms of the Somatic Discomfort cluster, while the performance decrement on short term memory tasks was found to be more highly related to symptoms of the Arousal Level cluster. If this result is found to be reliable in future studies, it would suggest that any method or agent designed to reduce mountain sickness would be effective (from the viewpoint of mental performance), only if it both reduced the Somatic Discomfort (e.g., NAUSEA, HEADACHE and DIZZINESS) and increased the Arousal Level (e.g., ENERGY, VIGOR, etc.) of subjects while at high altitude.

BODY OF REPORT

WORK UNIT NO. 083

Physiological and Psychological Aspects of Performance at Altitude

STUDY NO. 4

Effects of Codeine, Methysergide and Phenformin on Symptomatology and Performance at Altitude

PROBLEM:

The success of efforts to reduce the symptoms of Acute Mountain Sickness (AMS) depends partly on the development of reliable and valid means to measure the subjective symptomatology of AMS. The General High Altitude Questionnaire (GHAQ) Evans, 1966), a 22-symptom Likert scale, satisfies this requirement. Evans (1966) reported a high split-half reliability ($\mathbf{r} = .92$) for the over-all scale. The purpose of this study was to obtain additional information concerning both the validity of the GHAQ and to identify symptom clusters within the GHAQ leading to the development of GHAQ symptom sub-scales.

Both construct and predictive validity of the GHAQ were measured in this study, 1 To establish the construct validity, two other methods of assigning severity rating to AMS were used in addition to the GHAQ, namely, a paired comparisons technique and physician judgment,

Information concerning sub-scales of the GHAQ was obtained by performing key-cluster analyses (Tryon & Bailey, 1966) on the low and high altitude GHAQ protocols. The key clusters of symptoms from these analyses are being used to form the bases of independent subscales of the GHAQ.

This analysis was accomplished on data from a 1967 study reported in a previous Annual Research Progress Report. In all, 23 male subjects (18-23 years old) participated in this study. All subjects were given the GHAQ measures twice daily for nine days at a low altitude site (Fort Lewis, Washington; sea level) and five days at high altitude (Pikes Peak, Colorado; 14, 110 feet). Performance measures were given daily at low

¹Construct validity refers to the extent to which other methods of measuring the same construct (in this case, AMS) are in agreement with the measure of interest (GHAQ). Predictive validity of the GHAQ was established by regressing GHAQ symptoms scores to the mental performance measures (e.g., Reaction Time, Short-Term Memory, and Digit Symbol Substitution Test).

Physiological and Psychological Aspects of Performance at Altitude (Cont¹d)

and high altitude. Transfer from low to high altitude sites was accomplished for all subjects within a ten-hour period.

RESULTS

All GHAQ symptoms, except LIVELY showed a statistically significant change from low to high altitude (all sign test p's <, 05), HEADACHE showed the largest change, increasing from a mean of 1, 14 to a mean of 3, 26 (on a 3-point scale). At high altitude, physician judgment of AMS severity and a paired-comparison technique to measure severity of AMS correlated with the GHAQ total scores, 59 and, 51 respectively, Both of these correlations reflect the construct validity of the GHAO. In regard to predictive validity of the GHAQ, subjects were divided into three groups according to the severity of the AMS symptoms as measured by the GHAQ: minimaliy, moderately, and severely affected. Those subjects experiencing severe AMS (as measured by the GH(Q)showed sharp decrements on all performance tasks, while those subfects in the less affected groups showed small performance decrements, or none at ali. This result suggests that high altitude per se does not affect mental performance; it does suggest that mental performance is directly affected by severity of AMS. This finding also helps to estabiish the predictive validity of the GHAQ. Additionally, multiple linear regressions were calculated for all performance tasks to determine the ability of individual GHAQ symptoms to predict specific performances. The multiple R's used I to 3 GHAQ symptoms in the regression countion, and ranged from , 49 to , 80 for 10 mental performance measures with the median R being . 67. Such consistently high relationships between specific symptoms and the mental performance tasks indicate high predictive validity for specific symptoms for mental functioning. Finally, in regard to predictive validity, the GHAQ total scores factors all symptom scales) correlated highly with the mental performance.

In order to obtain information about sets of GHAQ symptoms that group together, key cluster analyses were calculated on the GHAQ protocols for iow and high altitude. At high altitude, which is of the most interest, two major clusters of symptoms were identified and given superordinate names descriptive of their member cluster symptoms, i.e., Arousal Level and Somatic Discomfort. A low altitude cluster of GHAQ symptoms, Mood, was also identified (See Table 1). Physiological and Psychological Aspects of Performance at Altitude (Cont¹d)

Table 1 🤟

Rey Cluster Analyses: Primary Sub-Scales of the GHAQ

r	ima	۲y	Sup-	• Sca	le.	Name	

GHAQ Component Symptoms

1. Arousal Level

Active Satisfied Comfortable Energetic Refreshed Vigorous

Short of Breath

Heart Pounding

Nausra

Dieev

2. Somatie Discomfort

Headache Happy Satisfied Pleased

3. Mood

The primary sub-scales (key clusters) may be used as the basis for refining the GHAQ to develop stable and valid sub-scales in future work. Such a development should produce a marked increase in the utility of the GHAQ to identify the primary components of the AMS syndrome requiring treatment in order to satisfactorily reduce potential performance decrements in mental functioning at altitude.

CONCLUSIONS

1. The GHAQ shows good reliability and validity as a means to measure Acute Mountain Sickness.

2. The GHAQ measures three separate components of subjective symptomatology: Arousal Level, Somatic Discomfort, and Mood.

RECOMMENDA TIONS:

1. Expansion of the three primary GHAQ sub-scales in luture studies using the basic format of the present study.

2. Continuation of a research effort directed at relating basic physical logical changes to the development of symptomatology as measured by

Physiological and Psychological Aspects of Performance at Altitude (Cont¹d)

the GHAQ.

3. Due to the need to effect better management of personnel and financial resources, research under this work unit will be discontinued. Future efforts of this work unit will be transferred in FY 70 to Work Unit 082, Metabolic, Physiological and Psychologican Effects of Altitude.

PUBLICATIONS:

1. Carson, R. P., W. O. Evans, J. L. Shields and J. P. Hannon. Studies on the Symptomatology, Pathephysiology and Treatment et Acute Mountain Sickness, Proceedings of the International Symposium on Altitude and Cold. Fed. Proc. 28:1085-1091, 1969.

2. Carson, R. P. Military-Medical Aspects of High Altitude, AGARD/NATO Symposium: Aeromedical Aspects of Troop Transport and Combat Readiness, AGARD Conference Proc. No. 40, Oct. 1968.

3. Evans, W.O., R.P. Carson and J.L. Shields. The Effect of High Terrestrial Environment on Two Different Types of Intellectual Functioning. Biomedical Problems of High Terrestrial Elevations. A. H. Hegnauer (Ed.), USARIEM, Natick, Mass., pp. 291-294, 1969.

4. Shields, J. L., J. P. Hannon and R. P. Carson. Pathophysiology of Acute Mountain Sickness. Biomedical Problems of High Terrestriai Elevations. A. H. Hegnauer (Ed.), USARIEM, Natick, Mass., pp. 24-31, 1969.

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23. (U) Tech Objective: 1) To elucidate myocardial changes and their causes in animals exposed to high altitude, 2) To describe the histological and chemical nature of these changes and 3) to determine whether these changes can be considered pathological or desirable adaptive processes.

24, (U) Approach: Animals are housed in heated quarters at 14, 110 ft. and at sea level or intermediate altitudes, to l) test the prevalent notion that cats do not acclimate to change in altitude, 2) compare the physiologic and pathologic response of altitude-exposed cats with our observations in other species, 3) investigate the changes in cardia and skeletal muscle after altitude exposure with biochemical measurements and light and electron microscopy 4) quantitate in monkeys cerebral blood flow, CSF pressure, brain water and constituents of blood and CSF during 1-5 days exposure to 14,110 ft. 25. (U) Progress (Jul 68 - Jun 69): Study 2 (cats) is complete. Cats native to 5, 380 ft. can successfully adapt to 14, 110 ft. for 3 months. Their hematologic and cardiovascula response is similar to that of rats and rabbits and not greatly different from that of dogs. Study 3, involving mitochondria from myocardium and skeletal muscle revealed Changes in both ultrastructure and metabolism; species differences existed as well. Descriptions of the ultrastructural changes have been published. Metabolically fatty acid oxidation increased 2-3 fold in dog hearts, in the rat 02 uptake decreased. Further of these changes is planned (Study 5).

Study 4, involving blood flow and intracranial pressure in mondeys, is in progress with 10 monkeys now at 5,380 ft., 30 at sea level. Publications are listed in the Annual Report. 188

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ABSTRACT

PROJECT NO.

3A061102871R

Research in Biomedical Sciences

TASK NO. 05

WORK UNIT NO. 085

Effects of Altitude on Myocardium of Animals

Environmental

The following investigations have been conducted under this work unit:

STUDY NO. 2 The Effects of Altitude, **Exposure Time and Species** on the Development of Hypoxic Cardiac Hypertrophy and Related Factors; Domestic Shorthair Cats

STUDY NO. 3 Effects of Prolonged Altitude Exposure on the Morphology and Biochemistry of Cardiac and Skeletal Muscle in Animals

- STUDY NO. 4 Effects of Altitude on the Cebus Monkey (Cebus apella) with Emphasis on Cerebrospinal Fluid Pressure and Brain Water Content
- STUDY NO. 5 Effects of Prolonged Altitude Exposure on Lipid Metabolism in Mammalian Cardiac and Skeletal Muscle

The general objectives of Studies 2, 3 and 5 are to characterize the effects of altitude on myocardium of animals and to correlate these where possible with physiological and subcellular functional changes. The cause of observed changes will be sought, and an attempt will be made to determine whether such changes are desirable adaptive phenomena or deleterious processes. In FY 68, rots, dogs,

and rabbits were compared to determine comparative effects of altitude and the influence of differing relative heart size on adaptation. In Study 2 the cat was surveyed for similar effects. This species was chosen because only inconsistent data were available relative to its performance at altitude, and to further broaden the basis from which to extrapolate to man. In Study 3 an observed morphologic change in heart mitochondria of three species was examined more closely to determine the type and degree of subcellular enzyme changes which were associated with it.

Groups of animals were exposed to 14,110 ft. (Pike's Peak), 5,380 ft. (USAMRNL) and sea level (USARIEM). Study 2 compared animals maintained at 5,380 ft. with those maintained at 14,110 ft. Study 3 compared the effects of altitude on animals maintained at sea level vs. 14,110 ft. In Study 2 hematologic parameters were recorded throughout the 3-month exposure period. Blood pressure and right ventricle blood oxygen saturation were determined before and after exposure to altitude. At necropsy hearts were weighed and total weights and component weights were recorded. Specimens of all organ systems were prepared for light microscopy and hearts, lungs, and kidneys were sampled for electron microscopic examination.

Procedures for Study 3 were similar; additionally, specimens of myocardium, diaphragm and skeletal muscle were analyzed for enzyme activity at altitude, to evaluate the functional integrity of mitochondria. Biochemical determinations included anaerobic metabolism of glucose ¹⁴C-6-phosphate by muscle homogenates, measurement of ATP, ADP and inorganic phosphate in extracts of muscle, oxidation of glutamate, succinate, and palmitate 1-¹⁴C by isolated mitochondria.

Study 2: Twenty adult domestic shorthair cats native to 5,380 ft. were transported to 14,110 ft. and held for 90 days in a heated laboratory trailer. Twelve control cats were maintained at 5,380 ft. After an initial week of anorexia and depressed activity, the animals remained healthy for the duration of the study. Compared to mean values for the controls and their own values at 5,380 ft., the animals at altitude for 90 days had increases of 29% in packed cell volume, 10% in hemoglobin, and 128% in right ventricular pressure. Total ventricular weight increased by approximately 10%, right ventricular weight by 28% and right ventricular wt/total ventricular wt ratio by 81%. Structurally abnormal mitochondria were present in hypertrophic myocardium. Oxygen saturation of right ventricular blood decreased by almost 23%. The females were considered to demonstrate greater adaptive responses than the males in all parameters measured. The cats were not unique in their response to altitude but were generally similar to rabbits and rats and not greatly different from dogs studied similarly.

Study 3: Rats, rabbits, and dogs were housed at 14,110 ft. for 3 months, with control animals at sea level. Abnormal mitochondria occur in the hypertrophied myocardium; they are irregular, swollen and vesicular with shortened, disorranged cristae in reduced numbers. Myocardial mitochondria from dogs so exposed had increased obility to axidize palmitate to CO_2 ; in those from exposed rats, oxygen uptake was reduced; those from rabbits, unlike dogs, had decreased obility to oxidize palmitate to CO_2 . In skeletal muscle, the only altitude-reloted changes seemed to be increased phosphate potential in mitochandria from rabbit semitendinosis and increased axidatian of palmitate in those from rat rectus femoris. This study was a collaborative effort among investigators af Pathology, Chemistry and Physialogy Divisions, USAMRNL; USARIEM, Natick, Massachusetts; and Department of Biochemistry, Kansas State University.

Study 4: Using Cebus apella monkeys and the hypothesis that increased introcranial pressure underlies many of the symptoms of "acute altitude sickness," this current study is an attempt to quantitate some of the mechanisms. Thirty monkeys are held at sea level (WRAIR) with 10 as control and 20 to be shipped to Pike's Peak (14,110 ft.) for 1-5 days' exposure. Holf will be given a diaretic. Measurements will include systemic and right ventricular blood pressure, carotid flow, O₂ and CO₂ tension and pH af blood and cerebrospinal fluid (CSF), CSF pressure, brain weight and water content, electron microscopy af brain. Ten additional monkeys are at MRNL and standardization of techniques is in progress.

Study 5: Rats, rabbits and guinea pigs will be used to confirm the observations of Study 3 with refinement of techniques. Three exposure periads: 2, 6 and 12 weeks will be used. Animals are on location.

BODY OF REPORT

WORK UNIT NO. 085

2

STUDY NO.

Effects of Altitude on Myocardium of Animals

Effects of Altitude, Exposure Time and Species on the Development of Hypoxic Cardiac Hypertrophy and Related Factors; Domestic Shorthair Cats

PROBLEM:

1. Right heart enlargement resulting from exposure to reduced atmospheric pressure has been demonstrated in man and several other species. The most popular theory to account for its occurrence is that it is a compensation for the increase in pulnionary vascular resistance usually observed at high altitude. Pulmonary arterioles undergo vasoconstriction due to change in vasomotor tone and/or anatomical changes in the vessel wall. The right ventricle must pump against greater resistance which results in an increase in force per unit crosssectional area of the muscle. To return the force per unit area to normal by increasing the total muscle area, myocardial hypertrophy occurs.

2. Previous work at this laboratory during the summer of 1966 and 1967 showed some species variation in response to prolonged exposure to high terrestrial elevations, i.e. although rats, rabbits and dogs all showed similar magnitudes of increase in both erythrogenesis and pulmonary arterial pressures, cardiac hypertrophy proved to be considerably less in the dog than in other species. Completion of Study No. 2 during the summer of 1968 showed that cats reacted with myocardial hypertrophy similar to rats and rabbits. However, in those species which readily develop cardiac enlargement, the nature of this change is incompletely understood. It has been suggested that the hypertrophy is related to actual fiber thickening but adequate data are lacking. Similarly, information of water and protein changes is inconclusive.

3. It appears worthwhile to continue this area of research to more fully explore the nature of these changes and determine more accurately to what extent they may be extrapolated to man.

4. While previous studies have been useful in describing changes occurring in several animal species subject to prolonged exposure to 14,110 ft., the problem of "acute mountain sickness," a transient varyingly-debilitating condition, is of considerable importance to military planners. It occurs during the first days (1-5) of exposure. Study No. 4, using Cebus monkeys, is designed to examine some physiologic mechanisms which may contribute to production of signs or symptoms of "acute mountain sickness." Rational prophyllactic or therapeutic measures can be based only on elucidation of such mechanisms (blood gas tension, blood pH and pressure, cerebral blood flow, cerebrospinal fluid pressure and gas tension, brain water content).

RESULTS AND DISCUSSION OF RESULTS: (Study 2)

Twenty adult domestic shorthair cats, native to 5,280 feet were transported to a heated field laboratory at 14,110 ft. on Pike's Peak summit, after a suitable period of laboratory conditioning (at 5,380 ft.). Twelve control animals were maintained at 5,380 ft. at the Denver facility.

Initially, all cats taken to altitude displayed signs of acute mountain sickness, i.e. lassitude and anorexia. An average 11% loss in body weight was noted at the end of the first week. However, within a month all animals appeared normal clinically and had regained their initial weight. The animals were housed at 14,110 ft. for 3 months.

One male cat died, with pneumonia and pulmonary edema, after 3 weeks.

Periodic blood samples revealed significant increases (P<.05) in packed red cell volume (PCV, %) and hemoglobin concentration (gm/100 ml) after 3 months exposure to 14,110 ft.:

Exposure to		
14,110 #	PCV	Hemoglobin
Day 0	37+5	14.3+2.1
Day 89	47 <u>+6</u>	15.8 <u>+</u> 1.8

Erythrocyte counts (RBC) also increased over the exposure period, proportional to PCV and hemoglobin, but erroneously low RBC counts on Day 0 preclude comparison of Day 0 and Day 89. Similarly, Day 0 RBC indices have abnormal

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values. A more experienced technician performed hematologic procedures during the latter portion of the experiment; erythrocyte count and red cell indices were toward the upper limit of normal by the end of the study:

Normal range (literature)	Da y 89
24 - 4 5%	47%
$5.0-10.0 \times 10^{6}$	8.5×10^{6}
8-15 gm	15.3 gm
³ بر ³	³ بر ³ 55.3
31-35%	32.4%
פעע 17-13	18 פעע,
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Peak right ventricular pressure (RV pressure, mmHg) more than doubled, comparing 12 of the altitude-exposed animals on which this pressure was determined with 8 from the 5,380 ft. group, while oxygen saturation of right ventricular blood (SO_2v) decreased significantly (11 and 7 cats, respectively):

Altitude	RV pressure	ΰO ₂ ν		
5,380 ft.	32 <u>+</u> 3	64+4		
14,110 ft.				
(90 days)	73 <u>+</u> 9	49+9		

Right ventricular hypertrophy was evident in ratios (%) of right ventricle/ total ventricular mass (RV/T) and similar ratios of left ventricle and interventricular septum/total ventricle mass (LV/T, S/T). The former was significantly increased, (P<.05) the latter two decreased (due to increase in total ventricular mass). Ratios of total ventricular mass and ventricular segments to body weight (BW) revealed significant (P<.05) increase in RV/BW and T/BW only (gm/kg): Mean ± 1 s.d.:

Aititude	RV/T	LV/1	<u>\$/</u> T	T/BW	ŔV∕₿W	LV/BW	S/BW
5, 380 ft.	23.59	42.02	34. 41	2.306	0.545	0.970	0.791
(n=12)	2.42	1.91	1. 94		.084	.114	.056
14,110 ft.	28.64	39.25	32.10	2.504	0.719	0.98 2	0.804
(n=20)	2.33	2.29	1.64	.262	.106	.106	.092

Histological examination of heart, lung, kidney, intestine, spleen, liver, and adrenals revealed no altitude-related lesions. However, electron microscopic studies did reveal ultrastructural changes in the myocardium, consisting of

swollen, disarranged mitochondria with irregular internal structure. In addition, swelling of myocardial capillary endothelial cells was observed.

CONCLUSIONS:

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Contrary to previous reports, this study indicates that domestic cats can, under favorable conditions, adapt to high terrestrial elevations although some clinical signs and I death were associated with the exposure. The difference between this and other studies was that our cats were maintained under controlled ambient temperatures during their stay at 14,110 ft. Cold as an additional stress may well be the factor which affected survival of cats in other studies.

The physiological changes observed in this group of cats were quite similar to those in rats and rabbits and not greatly different from these in dogs previously studied under similar conditions. It was of interest to note that in every parameter measured, the female cats appeared to adapt more successfully than the males.

RECOMMENDATIONS:

1. Additional long-term exposure data from subhuman primates would be useful to broaden our knowledge from which to extrapolate to man.

2. The effect of exercise on these changes should be evaluated.

3. An understanding of the pathogenesis of transient illness upon initial exposure to high elevations is needed. Study 4 is designed to explore some aspects of this area.

4. The functional significance of mitochondrial changes in hypertrophic myocardium of our animals requires clarification; Study 5 is designed to explore some aspects of this area.

STUDY NO. 3

Morphology and Biochemistry of Cardiac and Skeletal Muscle in Animals

STUDY NO. 5

Effects of Prolonged Altitude Exposure on Lipid Metabolism in Mammalian Cardiac and Skeletal Muscle

PROBLEM:

Does the morphologic alteration in mitochondria of hypertrophic myocardium (produced by altitude exposure) have functional significance? Investigators from Pathology, Physiology and Chemistry Divisions, USAMRNL, undertook to explore the question collaboratively under Study 3. Before the work was completed, investigators from the latter two divisions had relocated to USARIEM, Natick, Massachusetts and Division of Biochemistry, respectively. Final phase of the biochemical determinations was performed at the latter locations.

RESULTS AND DISCUSSION OF RESULTS:

Study 3 was a "feasibility" approach to the above question, comparing muscle metabolism in heart and skeletal muscle from dogs, rats, and rabbits housed at 14,110 ft. for 3 months, with similar material from sea level controls.

The ability of isolated heart muscle mitochondria to oxidize palmitate-1-14C to ¹⁴CO₂ was influenced by both the animal species and by the protonged exposure to altitude. Mitochondria isolated from both right and left ventricles of dogs exposed to altitude had an increased (P<.01) ability to oxidize palmitate-1-14C to 14CO2 compared to sea level controls (Table 1). Although oxygen uptake data were not collected from mitochondria isolated from the right and left ventricles of dogs, exposure to altitude had little effect on the phosphate potential and alycolysis data obtained from the right ventricles of dogs. Similar information from rat heart preparations indicated that altitude exposure had little effect on palmitate -1-14C oxidation rates, glycolysis data and phosphate potentials but significantly (P<.05) decreased oxygen ustake values. Preparations from rabbit myocardium, however, indicated a decreased (P<.05) ability of isolated mitochondria from "altitude exposed" rabbits to oxidize palmitate 1-14C compared to sea level controls. Oxygen uptake values, glycolysis data and phosphate potentials were similar for preparations from myocardium of rabbits exposed to altitude compared to sea level control animals.

TABLE 1

Effects of Altitude Exposure on Several Biochemical Parameters of Heart Muscle Metabolism¹

	Biochemical Parameters				
Species and treatment	Fatty acid oxidation ²	Oxygen uptake ³	Glycolysis	Phosphate potential ⁵	
Rabbit					
Sea level	6518+1382	57.5+24.6	. 38+. 12	.27+.11	
Altitude	2862+535*	80.8+17.1	. 43+, 12	. 22+. 18	

	Blochemical Parameters					
Species and treatment	Fatty acid oxidation ²	Öxygen uptake ³	Glycolysis	Phosphole potentia 1		
Rat						
Seo level	15,064+5000	53.5+20.5	.61+.31	.0502		
Altitude	18,576+5800	24.4-6.3*	. 407. 14	.04 .51		
Dog (right ventricle)						
Sea level	1452+731		.70+.4	. 32+ . 34		
Altitude	5061+1376**		. 3622	. 43 <u>-</u> . 50		
Dog (left ventricle)						
Sea level	2190+872		.62+.25	, 22+.17		
Altitude	61147892**		, 41 , , 14	. 43 45		

Animals exposed to altitude (14, 110 ft.) for 3 months. Data are expressed as mean \pm standard deviation. Values followed by a single asterisk are significantly (P<.05+* different and values followed by a double asterisk are highly significantly (P<.01)** different.

 2 dpm 14 CO₂/mg mitochandrial protein $^{-30}$ min produced from palmitate-1- 14 C.

 $^{3}\mu 1$ O₂ consumed/mg mitochondrial protein $^{\circ}$ hour; pyruvate substrate.

⁴ umoles lactate produced/mg homogenate N+30 min; glucose-6-phosphate as substrate.

⁵Total muscle concentration of ATP/ADP · Pi in µmoles/g.

Altitude exposure had little effect on these biochemical parameters (palmitate oxidation, oxygen uptake, glycolysis and phosphate potential) in preparations isolated from skeletal muscle of any species (Table II). There were two exceptions: exposure to altitude significantly (P<.05) increased the phosphate potential in rabbit skeletal muscle (semitendinosis) and also increased (P<.05) the ability of isolated mitochondria from rat skeletal muscle (rectus femoris) to oxidize palmitate-1-14C to $14CO_2$ compared to sea level controls.

TABLE II

Effects of Altitude Exposure on Several Biochemical Parameters

of Skeletal Muscle Metabolism¹

	Biochemical Parameters				
Species and treatment	Fatty acid axidation ²	Oxygen uptake ³	Glycolysis ⁴	Phosphate potential ⁵	
Rabbit (Semitendinosis)					
Sea level	623+380	33.3+13.5	. 40+. 17	. 14+. 11	
Altitude	1020 <u>+</u> 235	52.6720.0	. 407.20	. 54 <u>+</u> . 17*	
Rat (Rectus femoris)					
Seg level	531+77	42.5+21.0	. 60+. 46	. 36+. 17	
Altitude	1762+812*	41.5+2.0	.297.18	. 357. 14	
Dog (Sartorius)					
Sea level	1177+413	56.5+12.6	. 59+.28	. 74+ . 4 3	
Altitude	1757-888	59.34.2	. 497.27	1.04+.30	

Animals exposed to altitude (14,110 ft.) for 3 months. Data are expressed as mean <u>+</u> standard deviction. *Values followed by an asterisk are significantly (P<.05) different.

 2 dpm 14 CO₂/mg mitochondrial protein \cdot 30 min produced from palmitate-1 $^{-14}$ C.

 $^{3}\mu$ 1 O₂ consumed/mg mitochondrial protein \cdot hour; pyruvate as substrate.

 4μ moles lactate produced/mg homogenate N \cdot 30 min; glucose-6-phosphate as substrate.

⁵Total muscle concentration of ATP/ADP \cdot Pi in µmoles/g.

These observations suggest, from a biochemical standpoint, a similarity between the effects of altitude exposure on the myocardium of rats and rabbits and the effects of artificially induced heart failure in experimental animals. It may well be that the myocardial hypoxia experienced at 14,110 ft. is comparable to some phase of progressive myocardial hypoxia occurring during cardiac decompensation. It has

been reported both that mitochondria isolated from experimentally-induced failing rat hearts had lower oxygen uptake rates with several substrates compared to controls, and that experimentally-induced heart failure in guinea pigs did not influence the rate of oxygen uptake by heart mitochondria. There is a report that homogenates prepared from failing guinea pig heart had a decreased ability to oxidize fatty acids. It also indicated that failing guinea pig hearts had significantly lower concentrations of carnitine than control guinea pig hearts and that addition of carnitine to failing heart homogenates increased fatty acid oxidation to near control values. These observations suggest that failure in guinea pig hearts was manifested by a decreased ability to transport fatty acids from the extracellular space into the mitochondria where the fatty acids could be utilized for metabolism.

In our previous experiments, dog myocardium responded to the additional work load imposed by altitude exposure with increased rate of palmitate $-1-^{14}$ C oxidation to $^{14}CO_2$, thereby deriving increased amounts of energy. The increased work load was imposed not only on the right ventricle due to increased pulmonary vascular resistance but was apparently also imposed on the left ventricle (Table I).

RECOMMENDATIONS:

Study 3 was conducted during the summer of 1968. It is feasible to investigate further the metabolism of lipids by heart muscle in animals subjected to prolonged altitude exposure. Since there is considerable species variation in response to altitude exposure, it is of interest to survey the metabolic response of several animal species to altitude exposure. This recommendation forms objective for Study 5.

STUDIES:

Guinea pigs, rats and rabbits have been placed at 14,110 ft., with others scheduled to be transported there later; there will be 3 groups, representing exposures of 2, 6 and 12 weeks. A fourth group will be held at sea level (ARIEM), Natick, Massachusetts. This study is a collaborative effort among investigators of Pathology Division, USAMRNL: USARIEM, Natick, Massachusetts, and Department of Biochemistry, Kansas State University.

OBJECTIVES:

The general objective is to verify the results of Study 3 on biochemical alterations in lipid metabolism in the myocardium of animals subjected to prolonged altitude exposure. Marphological and physiological methods will be utilized to moritor altitude-induced alterations. Specific objectives include:

1. Monitor hematological and physiological parameters as a basis of comparison between species and also a comparison with previous studies.

2. Determine morphological alterations in heart and skeletal muscle by electron microscopy.

3. Determine the rate of aerobic oxidation of pyruvate $-1-^{14}C$, a-ketoglutarate-1- ^{14}C and palmitate-1- ^{14}C by heart and skeletal muscle mitochondria.

4. Determine the rate of aerobic oxidation of palmitate -1-¹⁴C by heart muscle homogenates and lysed heart muscle mitochondria.

5. Determine the concentration of carniting in heart and skeletal muscle.

6. Determine the gross composition (moisture, protein, lipid, glycogen, RNA and DNA) of heart and skeletal muscle.

7. Determine the concentration of serum-free fatty acids and serum glucose at termination of the experiment.

STUDY NO. 4

Effects of Altitude on the Cebus apella Monkey with Emphasis on Cerebrospinal Fluid Pressure and Brain Water Content

PROBLEM:

Humans native to altitude who travel above 12,000 ft. suffer a variety of transient symptoms including severe headache, lassitude, anorexia, nausea, oliguria, vomition, prostration and even pulmonary edema. While pulmonary edema is rare (i.e. 0.5% or less and seems to be associated with physical exertion and cold), nearly all have headache and some 20-40% have the remainder of the constellation of symptoms comprising "acute mountain sickness" or "acute altitude disease."

These symptoms regress with continued residence at the high altitude, with marked improvement occurring in 36-48 hours and virtual disappearance of symptoms by approximately 96-120 hours. (Onset occurs during the first several hours and symptoms are most severe 12-24 hours after arrival at altitude.)

Military operations at such altitudes with unacclimated troops would be hampered to the extent that this disease creates ineffectives, plus the requirement for medical personnel to see to those few wha could not care for themselves. Better understanding of the pathogenesis of the problem is required for rational prevention and management.

Pursuing the hypothesis that increased intracranial pressure is the common denominator for many if not all of the symptoms (ultimately, hypoxia is the basic change) monkeys will be used to quantitate some of the changes.

Ten C. apella monkeys, originating in Columbia, S.A. at altitudes less than 4500 ft. (and probably only a few hundred feet) were laboratory-conditioned at WRAIR, WRAMC (sea level) for 3 months after being held by an importer in Florida for 2-6 months. These animals will be sea level controls for base line values. Two similar groups of 10, also held at WRAIR, will be exposed to 14,110 ft. (Pike's Peak summit) for 1 to 5 days. These will be studied (2 per day) and sacrificed for tissue specimens. A fourth group of 10 has been housed at MRNL (5,380 ft.) for development of techniques, and for determination of values at that altitude.

Values of particular interest are pressure, pO_2 , pCO_2 , pH and base in cerebrospinal fluid; cerebral blood flow; systemic arterial and pulmonary arterial (or right ventricular) pressure; blood gases and pH; oxygen saturation; and brain water content (with electron microscopic examination of brain for possible compartmentation of such edema as may occur).

One of the groups of 10 altitude-exposed monkeys will be treated prophyllactically with a diuretic (furosemide) in an attempt to alter the changes anticipated in the untreated animals.

KESULTS AND DISCUSSION OF RESULTS:

The monkeys are now housed at WRAIR and MRNL. All have become laboratoryconditioned the very little problem. Routine TB testing and fecal examination for endeparasites has been conducted. TB tests have all been negative; nematode parasites, most notably Strongyloides species, were present, and treatment has been successful. Throat and rectal swabs from the MRNL group were cultured and Shigelia and Salmonella organisms, although expected, were not found. The animals do not have diarrhea or respiratory disease, eat well, and are gaining weight.

With phencyclidine (Sernylan[®], Parke-Davis) for chemical restraint, we are now adapting measuring techniques to the monkeys (i.e. spinal tap, catheterizations, placement of flow probes) and standardizing these methods. Some difficulty has been encountered in each instance but these should be overcome as experience increases.

Sea level measurements and sacrifice and the two sets of altitude exposures, will occur in July, 1969.

CONCLUSIONS:

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No conclusions with respect to altitude at this time. We have concluded, however, that the Cebus monkey is a very desirable laboratory animal. It was chosen because of freedom from Herpesvirus simiae ("B"), extremely low incidence of spontaneous tuberculosis, low altitude of origin and acceptable size (somewhat small perhaps).

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PROJECT NO.	3A061102B71R	Research in Bio-Medical Sciences
TASK NO.	05	Environmental Medicine
WORK UNIT NO.	090	Complex Cognitive Performance in Altered Environments

The following investigation is being conducted under this work unit:

STUDY NO. 1: Effects of Prolonged Exposure to Noise Upon Information Processing

The development of a set of behavioral tests designed to predict individual reactivity to prolonged environmental alteration is underway. During the past year, the work has centered upon construction of a Medical-Biographical Inventory designed to measure the perceived severity of past medical occurrences (e.g., operations, wounds, illnesses). Initial results indicate that a perceived severity index is related to preferred types of social-athletic activities. During the next year, the full set of measures of which the Medical-Biographical Inventory is a member, will be developed and their usefulness as a set to predict reactivity to the environmental alteration of prolonged noise exposure will be determined.

WORK UNIT 090

Complex Cognitive Performance in Altered Environments

STUDY NO. 1

Effects of Prolonged Exposure to Noise Upon Information Processing

PROBLEM:

There is wide variation between individuals in response to pain. discomfort, and stress or environmental alterations leading to pain or discomfort. For example, this variation has been repeatedly demonstrated for the subjective symptom reports of subjects exposed to high altitude. The severity of reported symptomatology has been demonstrated to be highly related to performance on both mental and psychomotor tasks; in general, the more severe the reported symptoms, the greater the performance decrements while at high altitude. Prediction of those individuals least and most susceptible (i.e., who report the least or most severe symptoms) would offer the capability to select the most satisfactory individuals for positions requiring exposure to environmental alteration. An assumption of this work is that no completely effective medical prophylactic or "cure" may be found for such conditions as Acute Mountain Sickness since a significant part of the symptoms may depend on the past experience with expectation of, and interpretation of the bodily and environmental cues experienced in the environmental alteration.

The development of a method to measure sensitivity to discomfort directly is a main object of this study. Discomfort thresholds for various sensory modalities (e.g., noise/tone, light, cold) will be determined by using the classical psychophysical techniques. An index score, for each individual (the mean deviation of the individual's thresholds for all modalities from the sample average) should be a measure of a general discomfort threshold. This index of the discomfort threshold is expected to relate to reactivity to environmental alteration.

RESULTS:

A system to measure the thresholds of discomfort on four modalities (i.e., cold temperature, noise/tone, muscle pain and electric shock) has been designed and is presently being fabricated. A Medical-Biographical Inventory has been developed to measure the perceived severity of past common medical occurrences such as minor Complex Cognitive Performance in Altered Environments (Cont'd)

operations, illnesses, and injuries. In pilot work, the index score of perceived severity has been found to relate to preferred sports activities (e.g., contact versus non-contact sports) and social activities (e.g., group versus solitary). The Medical-Biographical Inventory has been refined on the basis of these preliminary results.

CONCLUSIONS:

None

RECOMMENDATIONS:

1. Continuation of this program, which is presently in its initial stage, with the eventual object of developing a means to predict reactivity to environmental alteration.

2. Test the ability of the Medical-Biographical Inventory and discomfort thresholds index to predict reactivity (e.g., via subjective symptomatology) to a standard environmental alteration in the laboratory (e.g., prolonged exposure to noise).

3. Determine the generality of the usefulness of these procedures in predicting reactivity to various environmental alterations such as high terrestrial altitude, nutritional deficits and temperature extremes.

PUBLICATIONS:

None

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PROJECT NO. 3A062110A822

WORK UNIT NO. 065

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Military Internal Medicine

Microbiological Research in Tuberculosis

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The following investigations have been conducted under this work unit:

STUDY NO. 1 To Improve Mycobacteriology Laboratory Methods

STUDY NO. 2 To Develop Immunologic Methods for Detecting Humoral Antibodies to Mycobacterium tuberculosis

1. (a). The composition of the modified Middlebrook 7H10 OA medium, used for isolation of mycobacteria from clinical specimens has been modified by removal of penicillin (0.5 µg/m1) because of possible inhibitory effects on certain <u>M. tuberculosis</u> strains. The antibiotic was originally used to suppress chance contaminants which might have survived the decontamination procedures.

(b). Studies were made to determine the levels of p-aminobenzoic acid (PABA) which must be added to bloassay media to effectively eliminate growth inhibition effects of p-aminosalicylic acid (PAS) in sera from patients on multiple drug therapy.

(c). A reward in the continuing search for improvements to the system for "blue light" fluorescence microscopy was another barrier filter (Zeiss No. 53) which gives superior visualization of auramine O-stained bacilli.

Loss of fluorescence (non-retention of stain) by <u>M</u>. <u>tuberculosis</u> H37Rv cells in the presence of growth-inhibiting concentrations of isoniazid, and ethambutol parallels loss of acid-fastness for phenol-fuchsin stains.

(d). Serum inhibition titers on tuberculosis patients were performed with the patient's mycobacterial strain, M. tuberculosis H37Rv-Sensitive, and with and without p-aminobenzoic acid (PABA) in the bioassay medium. The H37Rv titer differed from that of the patient's organism in 54 per cent of 129 sera. In 26 per cent the H37Rv titer differed by more than 2 serial dilutions.

(e). A seemingly paradoxical situation involving low temperature storage of 7H10 drug-containing media is being investigated; storage at 4°C. apparently caused slight inhibition of growth initiation on drug-free medium

as compared with plates stored at room temperature (25-28°C.) The presence of congo red dye in this medium intensifies inhibition af growth. Media containing drugs must be stored in the refrigerator.

(f). Preliminary drug susceptibility studies on patient's M. tuberculasis on 7H10 OA agar containing 0.2, 0.5, and 1.0 μ g/ml af the new antituberculasis drug, rifampin, showed most isolates to be susceptible to 0.2 μ g/ml. The drug is stable for about 3 weeks in media stored at 4°C., but deteriarates rapidly at roam temperature.

(g). A spot test for detection of acetyl-isaniazid in urine was evaluated and found suitable far use by hospital ward personnel.

2. This study, temporarily suspended because af loss of the senior investigator and other technical persannel, will be resumed in July 1969.

WORK UNIT NO. 065

Microbiological Research in Tuberculosis

STUDY NO. 1

To Improve Mycobacteriology Laboratory Methods

PROBLEM:

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Definitive proof of mycobacterial disease rests in the isolation and identification of the etiologic agent from clinical materials. To provide useful information for the physician, accurate drug susceptibility data on the patient's mycobacterial isolate is necessary. Mycobacteria other than Mycobacterium tuberculosis cause disease, therefore the laboratory should be able to identify these species. Serum drug level assays and serum inhibition titers provide, respectively, information on the concentration and clearance rates of administered drugs and a measure of the antimicrobial effectiveness of the patient's drug regimen against his infecting organism. All procedures used must be carefully controlled and standardized; therefore, it is important that laboratory personnel have the knowledge and training to insure a high level of accuracy. The search for new procedures and better modifications of established procedures is a continuing process.

RESULTS AND DISCUSSION OF RESULTS:

New modifications and additions to mycobacteriology laboratory methods may be found in the forthcoming revision of USAMRNL Report No. 317, 10 May 1968, at which time copies may be obtained on request. The new revision should be available in early FY 70.

(a). Penicillin G has been routinely added to the stock albumin solutions, used in the Middlebrook 7H10 OA agar medium (final concentration 0.5 units/ml), to suppress chance contaminants which might have survived filtration procedures. The use of 2.5 units/ml penicillin G to liquid bioassay medium was found to decrease the minimum inhibitory concentration (MIC) of isoniazid (INH) for M. tuberculosis H37Rv-streptomycin (SM)-resistant from 0.04 μ g/ml to 0.03 μ g/ml. Because of possible inhibitory effects by penicillin in 7H10 isolation medium and/or synergistic action in drug-containing agar media, all penicillin has been deleted from these media. Initial experience has revealed no increase in the contamination rate.

(b). To determine serum drug levels (bioassay procedure) of patients on multiple drug therapy, either (1) the drugs not tested for must be suspended prior to the loading dose, (2) special test organisms which are resistant to the

other drugs in his regimen must be used, or (3) substances which destroy the effects of oth \sqrt{d} and $\frac{1}{2}$ must be added to the test system. The latter (3) is the most destrible of proach, and when used in combination with (2) these determination, allow, can be made without interruption of the patient's drug therapy. The addition of p-aminobenzoic acid (PABA) to the bioassay medium effectively eliminates the effect of p-aminosalicylic acid (PAS) in the patient's serum. The competitive ratio of PABA to PAS for sensitive tubercle bacilli is about 1:1, but increases to between 1:16 and 1:1024 with PAS-resistant strains. Varying the concentration of PABA (0, 2, 5, 10, 20, 50, 100 µg/ml) raised PAS MIC values in the tube dilution bioassay system from 0.03 µg/ml ml (No PABA) to 15 µg/ml (2 µg/ml PABA) to over 200 µg/ml with 20 µg/ml) (No PABA) to 15 µg/ml PABA. PABA concentrations of 20 µg/ml in the presence of 200 µg/ml PAS, which approximates the highest concentration expected in serum, allowed growth of M. tuberculosis H37Rv-sensitive. Thus, 20 µg/ml PABA is used in media for bicassays and serum inhibition titrations when it is desired to eliminate PAS effects. Earlier reports have recommended using 2 µg/ml PABA (Handbook of Tuberculosis Laboratory Methods, VA-Armed Forces Cooperative Study on the Chemotherapy of Tuberculosis, Washington, D. C. 1962, p. 32).

(c). The advantages of ultraviolet and blue light microscopy for examining auramine-stained mycobacteria are well documented. An additional barrier filter (Zeiss No. 53) which transmits wavelengths above 530 mµ, has been found useful in the system employing blue light for fluorescence microscopy of auramine O-stained mycobacteria and gives better visualization of the bacilli than is obtained with a barrier filter transmitting wavelengths about 500 mu. The greater contrast was afforded by the dark brown background and the yellow fluorescing organism as compared with a light green background with a barrier filter transmitting wavelengths above 500 mµ. Users of the blue light system should test both filters, for individual differences in visual acuity may be a factor in selection of the proper filter. Results of timed (2 minutes) re-examination with both filters of 126 auramine-stained smears from specimens, most of which had produced positive cultures with relatively few calonies, were not significantly different with regard to finding acid-fast bacilli, however a larger area of the slide could be scanned when using the 530 mµ filter.

Kubica and Dye (Laboratory Methods for Clinical and Public Health Mycobacteriology, U. S. Dept. HEW, NCDC, Atlanta, Ga., p. 23) stated as a disadvantage of the fluorescence method that "Organisms apparently dead or rendezed noncultivable by chemotherapy (and non-stainable by Ziehl-Neelsen Methods) may still be fluorescence positive". Studies were undertaken in this laboratory to compare the efficacy of the auramine 0-staining method with phenol-fuchsin methods to demonstrate INH- and ethambutol (EMB)-induced loss of fluorescence

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(acid-fastness). Growth endpoints in serum drug levels and MIC titrations, determined by loss af fluorescence in 50 per cent of the bacilli, were within experimental error of endpoints determined using 2, 3, 5- triphenyl tetrazalium chloride (TTC). A few auramine- and fuchsin-positive cells and many nonfluorescing (acid-fast) cells were seen in tubes wherein the INH concentrations were 6- to 8-fald greater than the MIC determined by TTC. Loss of acidfastness due to effects of INH and EMB was paralleled by loss of fluarescence. In accard with previous reports, streptomycin-treated bacilli lost neither acid-fastness nor fluarescence under these conditions. However, these studies did shaw that adequate caunterstaining with permanganate was very necessary for quenching the slight fluorescence imparted to other bacteria on the stained specimen.

(d). Serum inibitian (SI) titers are performed using the patient's mycabacterial strain and strain H37Rv-sensitive in a liquid bicassay medium with and without PABA. This serves to assess the total in vitro effect of his multiple drug therapy, campare the drug susceptibility of his organism with that of a standard strain, and evaluates the contribution of PAS to the overall inhibition titer. Hopefully, these studies will provide information on the effectiveness of chematherapy regimens.

SI titers were determined on 129 sera fram tuberculasis patients. Using either the patient's strain or strain H37Rv made no difference in the SI titer with 44% of patient's sera; the H37Rv titer was greater by one serial dilutian in 28% and one dilution lower in 5%. Titers greater by 2, 3, 4 or 5 dilutions with the Rv strain were seen in 22% of the cases, as cantrasted with 2% evidencing H37Rv titers lower by 2 or 3 dilutions than with the patient's mycabacterium. Additional studies to evaluate these differences are in progress.

(e). Heat labile drug-cantaining media are stared in the refrigerator to decrease drug deteriaration. In studies to determine the effect of storage time an drug concentration in 7H10 agar media it was noted that the size and number of colanies were greater on contral (no drug) quadrants of plates held at room temperature in plastic bags than an media stared in a like manner in the refrigerator. The effect became apparent in plates stored for periads greater than 3 weeks and was manifested by partial inhibition of growth initiation from small inocula. This inhibition of grawth was intensified by the presence of congo red dye (used as a marker) in the medium. Inhibitian due to the dye was nated also in plates stared at raom temperature, but was greater in the refrigerated plates. Plates with conga red in the cantrol quadrant were inaculated with M. tuberculosis H37Rv after storage for 4 weeks at the two temperatures; two woeks later growth appeared on control quadrants of media

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which had been stored at room temperature, but not on refrigerated media. Also, there was less growth on congo red media in plates stored at room temperature thon on non-dye containing media. Marker dyes have been removed from all drug susceptibility media and studies ore in progress to determine the cause of growth inhibition due to storage at low temperature.

(f). In anticipation of the future use of the antituberculosis drug, rifompin, direct drug susceptibility studies were performed by inoculating smeor-positive specimens on 7H10 OA agar containing 0.2, 0.5, and 1.0 μ g/ml rifampin. All of the isolates were susceptible to the 0.2 μ g/ml level of drug. In addition, a series of plates containing these concentrations of drug were stored at 4-6°C and room temperature (25-28°C) and periodically tested with a rifampin-susceptible H37Rv strain. Refrigeroted plates stored up to 3 weeks showed no evidence of drug loss (growth in the 0.2 μ g/ml quadrant), while plates stored at room temperature for one week allowed growth of occasional colonies. Plates held at room temperature for 2 weeks, inoculoted, then incubated, at 36°C for an additional 2-week period showed growth of about 5% of the inoculum on the 0.2 μ g/ml quadrant as compared with the drug-free control. Longer incubation (for growth of M. tuberculosis) resulted in growth of 50% of the inoculum, indicating a very rapid destruction of the drug at 36°C.

(g). Tuberculosis wards need tests by which ward personnel con determine whether patients regularly take their oral drugs, i.e. isoniazid (INH). For isoniazid, the best method is to chemically test for INH metabolites (isonicontinic acid, acetyl-INH) in the urine. Most of the methods reported are either too complicated for use outside the loboratory, produce noxious fumes, or lack adequate sensitivity or specificity. A safe and relatively simple spot test for a cetyl-INH in urine (Eidus, L. and Hamilton, E. J., Am. Rev. Resp. Dis., 89:587, 1964) was evaluated for accuracy and adaptability for use by non-laboratory personnel. The reagents employed were 10% solutions of KCN and Chloramine T which could be dispensed from medicine dropper bottles to disposable, white plastic spot test wells containing the urine specimen. Preliminary tests revealed one false positive (recorded as "trace") among 29 negative urines and 100% correlation with 30 urine specimens from patients receiving INH. Acetyl-INH, the principal metabolite of INH appearing in the urine, could be detected within one hour ond 6 hours (limits tested) after the loading dose. Reagents maintained potency up to a week under refrigeration. Small, amber, prescription dropper bottles containing the dry chemicals could be furnished the ward for reconstitution and use when needed. Utilization of the test in outpatient clinics by clinic personnel is also feasible.

Microbiological Research in Tuberculosis (Cont'd)

CONCLUSIONS:

The addition of new methods and modification of existing procedures provide data which are meaningful and are of value in the clinical management of tuberculosis patients.

RECOMMENDATIONS:

Studies to improve and expand mycobacteriology laboratory methodology should continue indefinitely.

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PROJECT NO. 3A062110A822

WORK UNIT NO. 066

Military Internal Medicine

Nilscellaneous Microbiological Clinical Research

The bacteriol variant L form and species of mycoplosma other than the recognized primary pathogens are being studied to evaluate their roles in clinically-recognized disease and in healthy symptom-free individuals. Humans, domestic animals, and laboratory animals are being observed. The role of the L forms and sub-species of mycoplasma in disease is not well defined nor understood. Clinical studies have proven very difficult to correlate disease states with the L forms isolated and a more basic approach has been incorporated in our studies. The methodology developed in these studies can be directly applied to clinical studies. The laboratory has established a complete capability for the isolation and identificotion of all human and some avian and animal strains of Mycoplasma and functions as a consulting laboratory to various military and civilian medical facilities.

WORK UNIT NO. 066

Miscellaneous Microbiological Clinical Research

PROBLEM:

To determine the role of Mycoplasma and bacterial variant L forms as etiologic agents associated with acute and chronic disease. The incidence and possible significance of Mycoplasma species other than the recognized primary pathogens are being studied and evaluated in clinically recognized disease and in apparently healthy symptom-free individuals. Methodology for the isolation and identification of mycoplasma is well established and reproducible. With two exceptions, the methods employed can be cansidered rapid (3-5 days). These two exceptions, Mycoplasma pneumoniae and the more recently described T-strains, still defy the desired rapidity of isolation and identification. The companion L form study continues to pose many complex problems. First, no standard methodology has been developed for the routine isolation of L forms. Secondly, the clinical conditions studied have proven very difficult to correlate with the L forms isolated. A more basic approach has been incorporated in our studies in that we are attempting to produce L forms of well-described pathogens, such as M. tuberculasis, Klebslella pneumoniae, Staphylococcus aureus, etc. and to evaluate their pathogenicity in laboratory animals. Problems have been encountered in this approach also. Not all strains of a particular species are susceptible to L form production. For example, we were able to produce L forms fram only one of four strains of staphylococcus studied. It is expected the methodology developed in these studies can be directly applied to clinical studies. Our approach to the Mycoplasma-L form study can be divided into three areas of emphasis.

1. Cooperative studies with the clinical services. All patients, adult and pediatric, on the Medical Service who have a proved or clinical diagnosis of any of the following entities are included in the study:

a. Collagen disease to include rheumatic fever, glomerulonephritis, disseminated lupas erythematosis, ond rheumatoid arthritis and its variant Reiter's disease.

b. Lymphomas to include Hodgkin's disease, reticulum cell sarcoma, giant follicular lymphoma, lymphosarcoma and chronic lymphatic leukemia.

c. Idiopathic pleural effusions.

2. Surveys. Large well-defined groups are being studied to determine the incidence of mycoplasma in:

Miscellaneous Microbiological Clinical Research (Cont'd)

- a. Normal urine
- b. Venereal disease patients
- c. Sputo from tuberculosis patients

d. Sputa, or throat washings from patients with acute respiratory disease of viral etiology

e. Sputa from patients with chronic non-TB respiratory disease.

3. Laboratory studies to develop methodology and support acute clinical problems in man, animals and birds.

RESULTS AND DISCUSSION:

Since the initiation of this project 182 patients have been studied for L forms. Twenty-five were positive for L forms and B are presently under study. The mycoplasma have proven to be much more ubiquitous than suspected. Urine samples were collected from 500 "normal" individuals and cultured for mycoplasma with 10.5% positive for M. hominis. Urethral swabs from 300 individuals being examined for venereal disease were cultured for mycoplasma with 54% of the specimens positive. These two studies are the subject of reports now in preparation. Unidentified species of mycoplasma have been isoloted from turkeys, turkey eggs, monkeys and cows. Several individuals have teceived training in mycoplasma methodology and seminars have been presented by the staff at local hospitals and universities.

CONCLUSIONS:

It has been demonstrated that L forms and mycoplasma can be isolated from a variety of clinically recognizable diseases. The relationship is not clear. The results obtained in the surveys do indicate, however, that some species classed as normal nonpathogens may be of an opportunistic nature in chronic respiratory diseases and further studies to explore this possibility are in progress.

RECOMMENDATIONS:

The current emphasis will be continued and expanded. A need still exists for more rapid diagnostic methods. One such method which may lead to more rapid and specific identification will be explored. This method will utilize

Miscellaneous Microbiological Clinical Research (Cont'd)

the pryolysis-gas-liquid chromatograph technic to determine some of constant structural factors which are species specific and unique.

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PROJECT NO. 3A062110A822

Military Internal Medicine

WORK UNIT NO. 067

Computer Classification of Pulmonary Disability

The following investigations have been conducted under this work unit:

- STUDY NO. 1: Remote Control Data Transmission-Receiving for a Digital Computer System.
- STUDY NO. 2: A Biomedical Information System for Pulmonary Disease Services
- STUDY NO. 3: The Classification of Pulmonary Disability Through a Computer Based System

The development of Computer-Communication using a data terminalcomputer linkage approach between Valley Forge General Hospital and the Digital Computer System, U. S. Army Medical Research and Nutrition Laboratory is underway. The implementation of this system is currently taking place. The data display unit is currently under test and evaluation. This data system will the in with the information system developed for the Pulmonary Disease Service, FGH.

-A biomedical information system is being designed and programmed, in cooperation with the Pulmonary Disease Service, for a general purpose digital computer. This system is designed to process under automatic controls the information functions of data collection, storage, retrieval, analysis and display. The basic components (sub-systems) of the Master System are the specialized and related items of clinical information, laboratory data and medical reasoning foundation utilized by physicians in providing health care services to patients with pulmonary diseases.

A Classification Technique is being developed to simultaneously handle the qualitative data derived from clinical evaluation and quantitative data derived from physiological evaluation. The Classification Technique is now capable of producing, on s daily basis, a classification of Pulmonary Function status on patients studied on the Pulmonary Disease Service, Fitzsimons General Hospital.

To implement each of the studies, certain basic programs involving the storage, retrieval, analysis and display of information must be developed. These programs are used in all the projects. Examples of this type of programming are mathematical techniques (function minimization, linear programming), statistical (least square polynomial, T-test) and file manipulation (data retrieval, translation from natural language to machine language).

WORK UNIT NO. 067

Computer Classification of Pulmonary Disability

Remote Control Data Transmission-Receiving for a Digital Computer System

PROBLE.1:

STUDY NO. 1

To establish a computer based Pulmonary Disease Information System which can be accassed for information storage and retrieval by a qualified user who is not in the same geographical location as the computer center.

RESULTS AND DISCUSSION OF RESULTS:

The computer programming has been authored and debugged to accept information generated by the Pulmonary Disease Service, Valley Forge General Hospital, and store this data in the patient master file. Spirometry and lung volume calculation are currently being performed and the results stored on the patient master file. Medical histories (smoking, respiratory disease and Form 38) are also being collected and stored on the patient master file. This collected data is used to provide the information for the CIR (Clinical Information Report) program and the statistical evaluation program.

The transmission system has been installed, tested and accepted from the manufacturer. Active transmissions are taking place.

CONCLUSIONS:

1. It is feasible to support an installation geographically separated from the computer center.

2. Dedicated transmission facilities are required to insure error free information.

RECOMMENDATIONS:

1. Program development should be continued.

2. Effort should be made to acquire dedicated communication circuits.

STUDY NO. 2

A Biomedical Information System for Pulmonary Disease Service

Computer Classification of Pulmonary Disability (Cont'd)

PROBLEM:

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A tremendous amount of medical information is generated through the Fulmonary Disease Service (T.B., Non-T.B. and Fulmonary Function Clinic). For effective utilization of the data collected, a digital computer based information system is under development.

RESULTS AND DISCUSSION OF THE RESULTS:

Programs have been written for the computation, file storage and display of data collected by the Fulmonary Function Clinic. The data includes spirometry, lung volume (helium, nitrogen and body plethysmograph) and CO diffusion (gas chromatograph). Information generated from these calculations are returned to the physicians on a daily basis.

A system for the analysis, storage and feedback of data collected by physicians engaged in the diagnosis and treatment of tuberculosis is also being developed. The same procedures are used for the storage of the generated information as in the other information projects. Information units collected in cooperation with the T.B. section are:

- 1. Tuberculosis section conference
- 2. Current therapy
- 3. Initial therepy
- 4. Admission and contact
- 5. Respiratory history
- 6. Admission physical examination
- 7. X-ray findings
- 8. Thoracic surgery
- 9. Discharge data

There are three information units collected from the Microbiology Division, USAMRNL, which are:

- 1. Initial culture entry
- 2. Completed culture results
- 3. Serum drug levels

These information files are being combined to form a summary for the use of the physician in his evaluation. The data filing, done in connection with Microbiology, replaces their manual system of storage and retrieval of the information.

A sub-unit of the information system is the analysis of data to determine the effects of concentration and volume variation upon T.B. skin testing.

RECOMMENDATIONS:

Development of this information system should be continued.

Computer Classification of Pulmonary Disability (Cont'd)'

PUBLICATIONS:

Syner, J. C.; A Computer Based Biomedical Information System. I. Logic Foundations and Techniques USAMRNL Report #320 October 1968.

STUDY NO. 3

The Numerical Classification of Pulmonary Disability Through a Computer Based System

PROBLEM:

To develop a classification system of pulmonary disability based on a mathematical-logical process implemented on a digital computer.

RESULTS AND DISCUSSION OF THE RESULTS:

The classification has been approached from two avenues, that of statistical and mathematical manipulation of collected data for the physician to use in his evaluation and the use of techniques of numerical classification using Boolean (0,1) functions to have the computer evaluate the degree of pulmonary disability.

A statistical classification procedure has been implemented, in connection with the information system, to evaluate air volume and air flow functions reflected by the spirometry tracing. This procedure may be requested by the physician for his evaluation of his patient or for the evaluation of groups of patients.

The Boclean classification procedure has not been under active development because of the departure of the primary investigator.

CONCLUSIONS:

The statistical and mathematical manipulation of the collected data provides the interested physician with an additional tool in his evaluation of pulmonary disability.

RECOMMENDATIONS:

1. Development of the ability to perform mathematical and statistical evaluations and manipulation of the collected data be continued.

2. The attempt at Boolean classification should be suspended until an appropriately trained investigator is assigned.

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PROJECT NO.	3A062110A822	Military Internal Medicine
WORK UNIT NO.	068	Computer Instrument Linkage

The following investigation has continued under this work unit:

STUDY NO. 1: Continuous Oxygen Consumption under steady state exercise.

An automated system to affect the digital handling of analog instruments utilized in clinics and laboratories is under study and development in the U. S. Army Medical Research and Nutrition Laboratory. A system has been developed for the storage and retrieval of data messages derived from field studies. Developmental work is progressing on the handling of automatically acquired data from the measurement of oxygen consumption in humans.

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WORK UNIT NO. 068

Computer Instrument Linkage

STUDY NO. 1

Continuous oxygen consumption under study state exercise

PROBLEM:

To develop the electronic and digital computer programming systems required to handle the processing, storage, and retrieval of data derived from energy expenditure studies of subjects undergoing exercise on motor driven treadmills and bicycle ergometers.

RESULTS AND DISCUSSION OF THE RESULTS:

An "analog to digital data conversion system" has been established within the Bioenergetics Division and is being field tested with a ten channel "continuous oxygen consumption analysis system" (USAMRNL Report No. 318) in parallel with a conventional analog strip chart recording. This data handling system will be composed of several subsystems which will eventually carry the data from the recording instruments to final oxygen consumption data with a minimum of manual intervention.

The system will be subdivided into subsystems:

1. Instrument to "analog to digital data conversion system" linkage and digital data recording on punched paper tape.

2. Sorting, gross editing and storage of raw data as millivolt signals in digital form.

3. Retrieval and selective listing of raw data as millivolt signals.

4. Retrieval, scaling, reduction, listing, and storage of data as gas analysis parameters.

Computer Instrument Linkage (Cont'd)

5. Calculation, selective listing and storage of oxygen consumption data.

6. Establishment of data files related to the energy expenditure files.

7. The on-line linkage of the "continuous oxygen consumption analysis system" directly to a digital computer.

As exploratory work progressed on the sorting, editing, and storage of raw data it became obvious that a format must be established for the storage and retrieval of the find gas parameter data. Therefore, attention has been focused on the establishment of data files which can be retrieved in a meaningful manner. This phase of the system (subsystem no. 6) has been developed. Data collected from field studies has been utilized as test data and is studied in conjunction with oxygen consumption parameter. Our established procedure allows the filing of data messages by the social security number of the subject being studied and the selection, retrieval and listing of stored data. Data can be retrieved, for example, by groups determined by field study, age, data range, etc. The capability to digitally record scanned analog data on punched paper type (subsystem no. 1) is well established while methods of sorting, editing and storing raw data on magnetic files (subsystem 2) has required constant updating and modification. These changes have been dictated by the manner in which the digital data is recorded and the way in which the data will be used in the subsequent subsystems. Therefore, subsystem 2 is undergoing major rewriting while the subsystems dependent upon it are planned.

CONCLUSIONS:

The automatic handling of oxygen consumption data is indeed feasible but the digital computer programming must be extremely flexible to allow manual intervention in conjunction with the editing of data. At the same time, personnel involved with the original data collection must be appraised of the need for self-discipline in the rigors of instrument use.

RECOMMENDATIONS:

Developmental work should continue on means of editing and storing the automatically collected raw data, allowing for manual intervention in the editing steps. Study should be made of the data to be retrieved from the system along with its relationship to other physiological type data and the data messages developed which will lead to meaningful data retrieval.

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PROJECT NO. JA062110A822

WORK UNIT NO. 071

Military Internal Medicine

Intravenous Fat

Emulsions STUDY NO. 1 To Produce a Non-toxic Intravenous Preparation Which will Provide a Relatively High Caloric Intake per Milliliter of Solution for Patients Unable to Assimilate Sufficient

Nourishment

Commercial and experimental fat emulsions were administered intravenously to rats and rabbits and the animals evaluated for toxic effects.

One large group of rats was given soybean oil emulsion, or components of the emulsion, with some subgroups also receiving antioxidants or lipotropic agents. The treatments were carried out by Dr. H. C. Meng, Vanderbilt Univ., Nashville, Tenn. Tissues were evaluated at USAMRNL. Animals receiving the lipid developed microgranulomas in spleen and liver, as well as pigment deposition in these areas. Incorporation of tocopherol with the emulsion markedly increased these effects; while oral methionine or tocopherol had little effect.

Two cottonseed oil emulsions were administered intravenously to rats and rabbits at USAMRNL. Rats and rabbits developed the pigment and microgranulomas in liver and spleen, from either emulsion. The rabbits had transient hyperpyrexia following infusion (5 days per week, for 3 weeks). Hematologic abnormalities did not occur.

In a third study, rabbits were sacrificed at intervals of up to 3 years following completion of infusions of Lipomol, a cottonseed emulsion. Tissues from these animals are now being evaluated.

WORK UNIT NO. 071

STUDY NO.

Intravenous Fat Emulsions

To Produce a Non-toxic Intravenous Preparation Which will Provide a Relatively High Caloric Intake per Milliliter of Solution for Patients Unable to Assimilate Sufficient Nourishment

PROBLEM:

To evaluate the effects of intravenous administration to animals of lipid emulsions, emulsifier systems, lipotropic agents and antioxidants alone or in combination.

RESULTS AND DISCUSSION OF RESULTS:

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The major portion of this report is devoted to tabulation of histologic findings in livers and spleens of rats. These animals were treated with the various products by Dr. H. C. Meng, Vanderbilt University, Nashville, Tennessee. They received, as a complete emulsion, a 20% soybean oil preparation (Intralipid[®]), 6 gm/kg/day. Other groups received only emulsifiers, only normal saline (PSS, 0.9 NaCl) or antioxidant, and lipotropic agents alone or in combination.

The only microscopic change of significance was the presence of a characteristic "intravenous fat pigment" in the reticuloendothelial system and formation of small granulomas. Both changes were most evident in liver and spleen.

To evaluate these changes, specimens of liver and spleen were submitted by Dr. Meng to MRNL where the tissues were processed, stained and read. The tissues of each rat were evaluated independently by three investigators, using replicate slides from the same tissue block. They rated each section with the following scale:

	Ø	3	2	3	4	5
Amount of Pigment:	none	slight	mild	moderate	marked.	iextensive
Microgranulomas:	None	rare	Few	Many	Vabundarit	humercus

Both organs of each animal were scored by each of the three investigators. The resultant grades were then averaged to yield a single grade for each slide (organ).

Intravenous Fat Emulsions (Cont'd)

Table 1 is a summary of the group mean score for each slide. Inspection of Table 1 reveals the difference in occurrence of microgranulomas and pigment. These numbers lend themselves to comparison of treatment means using Student's "t" test, but the precision of the statistical method exceeds by far the accuracy suggested by the mean scores. The treatment groups are small and varied in number and there is considerable variability in the data; this and the necessary averaging renders "statistical significance" of doubtful use. Further, degree of sampling error is unknown, i.e. we can only assume that the sections examined were representative. a sustain the statement of the state of the second statement of the statement of the second statement of t

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Summary of Microscopic Findings of Sections of Livers and Spleens (Mean Scores for Treatment Groups)

	101	Microg	anutoma	Pigment		
Treatment	Rats	Liver	Spleen	Liver	Spleen	
Intralipid (atome)	17	0.23	1.29	1.18	2.29	
Emulsitier X85012	マ	Ś	0.43	0.71	0.86	
Emulsifier X65013	3	Ś	1.20	1.00	1.40	
PSS + Methicnine 100 mg	8	Ø	0	Ś	0	
PSS + Vitamin E 5 mg	3	0	Ś	Ó	Ś	
PSS + Vitamin E 2.5 mg	2	Ó	i Ò	V	0	
PSS (atome)	3	Ø	Ø	0	Ś	
Intratipid + Selenium 250 µg	10	0.30	1.30	1.80	2.60	
PSS + Selenium 250 Mg	ۍ	Ś	Ś	Ś	0.33	
1955 + Setenium 100 mg	3	٥.	ø	(Ó	Ŵ	
Methionine 100 mg						
Intratipid + Vitamin E 2.5 mg	5	Ś	Ŵ	0.50	1.80	
Intratipid + Vitamin E 5 mg	۰ð	Ś	1.33	1.50	2.50	
Intralipid + Vitamin E 2.5 mg Methionine 100 mg	3	Ŵ	0.33	1.00	1.00	
PSS + Vitamin ES mg	2	Ó	Ø	0.50	1.00	
Wethionine 100 mc						
it + Vitamiri E 2.5 mg	4	Ŵ	. 3	0.50	0.75	
Intralipid + Methionine 100 mg Methionine 100 mg	15	Ø	1.00	1.00	2.20	
Introlipid + Vitemin E 5 mg	ъ	٥	1,17	I.3 3	2.33	
Intratipid + Selenium 100 µg	ৰ্ম্	0.165	1.15	1.18	3.80	
IntraTipid + Vitemin E (?) Vitemin E (?)	শ্	3.17	4.17	2.88	4.00	
Intralipid + Choline 100 mg	10	3.30	3.70	2.30	3.90	

Intravenous Fat Emulsions (Cont'd)

CONCLUSIONS:

The Banker of Street Hill

1. Intralipid (20%) results in pigment deposition and formation of microgranulomas in rat spleen and liver.

2. Emulsifiers alone result in fewer reactions than Intralipid.

3. Introlipid with Vitamin E incorporated in the emulsion results in more pigment and microgranulomas than Intralipid alone.

4. Intralipid and Vitamin E at either 2.5 or 5.0 mg/day is not much different from Intralipid alone.

In another study, tissues from rabbits given highly purified cottonseed oil emulsion intravenously were evaluated. These were 20% emulsions of absorbent fractionated oil stabilized with egg yolk phosphatide (Emulsions S. R. 207 and S. R. 208), each subjected to different but controlled amounts of oxidation during preparation. The rabbits (6 for each emulsion) were given 15 ml/kg/day, 5 days per week for 3 weeks.

During the infusion period, blood and urine values were within normal limits, rectal temperatures increased after infusion to ranges of 104.6 to 106.4°F but returned to preinfusion levels by the following morning. Other reactions are listed below.

	SR207	SR208	
Food intake	decrease, 30-70%	decrease, 30%	
Water intake	decrease, 25%	decrease, 25%	
Body weight, week 1	slight loss	slight loss	
Body weight, week 2	slight loss	slight gain	
Body weight, week 3	very slight gain	slight gain	
Body weight, week ends	very slight gain	gain	
Microgranulomas, liver	*	-	
Microgranulomas, spleen	*	*	
Microgranulomas, lymphoid appendix	*	*	
Pigment deposition, liver	*	*	
Pigment deposition, spleen	*	*	
Pigment deposition, lymphoid appendix	*	*	

Both products were also given intravenously to rats at the same dosage as the rabbits. While the rats made better weight gains during the infusion period, they also had microgranulomas and pigment deposition in livers and spleens. Both products also resulted in transient inflammation at the sites of injection (tail vein for rats, ear vein for rabbits).

Intravenous Fat Emulsions (Cont'd)

When received, both products had visible oil droplets suspended in the emulsion, on the surface and adherent to the inside of the container.

CONCLUSIONS:

SR207 and SR208 are unstable, result is systemic disturbance (abnormal weight gain in rabbits, hyperpyrexia) produce microgranulomas and intravenous fat pigment.

In a third study, 40 rabbits were given Lipomol intravenously at a dose rate of 50 ml per day, 5 days/week for 8 weeks and sacrificed at intervals up to 3 years thereafter. Liver biopsies were taken during the early post infusion period. These tissues are currently being analyzed.

PUBLICATIONS:

Abstract submitted for presentation at Internat. Soc. Parenteral Nutrition, Prague, Czechoslovakia, Sept. 1969: Meng, H. C., Jones, L. D., Ackerman, L. J., Fairchild, D. G., Bucci, T. J., "Studies of Long Term Administration of a Fat Emulsion in Rats Receiving Anti-Oxidants and Lipotrophic Agents."

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PROJECT NO.	3A062110A822	Military Internal Medicine
WORK UNIT NO.	072	Studies in Human Nutrition

The following investigations have been conducted under this work unit:

STUDY NO. 1	Estimation of total body stores and tissue concentrations of vitamin A in human subjects.
STUDY NO. 2	Preparation of generally labeled ¹⁴ C-beta-carotene for use in human vitamin A studies.
STUDY NO. 3	Experimental scurvy.
STUDY NO. 4	Further studies on the vitamin B ₆ requirement of young adult male humans.
STUDY NO. 5	Other nutrition activities, national and international

1. Studies on the liver and tissue concentrations of vitamin A in U.S. population groups have not been conducted recently. Livers from subjects in lowa (34) and Ohio (54) were analyzed for total vitamin A. Average concentration, was 122 µg of vitamin A/g liver. In either group there were 29% with concentrations of less than 40µg/g. This proportion of low liver vitamin A concentration levels is in agreement with the 32% found in a recent Canadian survey. Other tissues were found to cantain about 1µg/g tissue regardless of liver concentration. It has not been determined whether the high proportion of low liver stores were due to inadequate dietary intake of vitamin A or to other causes.

2. Generally labeled ¹⁴C-beta-carotene was prepared biosynthetically utilizing the mold <u>Phycomyces blakesleeanus</u>. The organism was grown in a defined glucose-acetate medium until the pH dropped to about 3. The cells were then centrifuged and resuspended in a mineral salts medium containing sodium acetate-1, 2-¹⁴C. Forty eight hours later, the cultures were harvested and the beta-carotene was extracted and extensively purified. Three separate runs were made and each run produced 10-20µC of pure beta-carotene with a specific activity of 7-21µC/mg. The yield of pure beta-carotene ¹⁴C was 0.1 to 0.2%. The beta-carotene produced was of sufficient specific activity for use in human studies.

3. A second study was conducted on the production of experimental scurvy in a group of six apparently healthy volunteers from the lowa State Penitentiary who were haspitalized on the metabolic ward of the University Hospitals, University of Iowa Medical School. They were fed the same diet as was employed in the initial study on scurvy and were labeled with 1-14C-4-3H ascorbic

Studies in Human Nutrition (Cont'd)

are a gas is -si-statistical is the subject of the subject of the

acid. One man withdrew whereas the remaining five completed the study. The data obtained confirmed and extended the conclusions derived from the initial experimental scurvy study.

4. A study employing human young adult male subjects was conducted to evaluate military "C-rations" in terms of their adequacy in vitamin B₆. The study was designed to provide additional information concerning the stability of vitamin B₆ in the ration and an estimation of the human requirement for this vitamin when the daily intake of protein is in excess of 150 grams. The data have been collected and are presently being evaluated.

5. Continuing support was provided national and international nutrition agencies and their basic or applied nutrition programs and training activities. Of particular significance has been the support provided the national nutrition surveys being conducted by the U.S. Public Health Service.

WORK UNIT NO. 072

STUDY NO. 1

Studies in Human Nutrition

Estimation of total body stores and tissue concentrations of vitamin A in human subjects.

PROBLEM:

The evaluation of vitamin A nutriture is difficult except in cases of frank vitar.in A deficiency. Reliable estimates can be obtained from liver concentrations of this vitamin, but the only practical means for obtaining such data are from autopsy samples.

Prior to 1950 over 20 studies on human liver vitamin A concentrations were reported from Western Culture countries. Mos. of these have been reviewed by Moore (1957) and Kirk (1962). More recent studies were reported by Smith and Malthus (1962) and by Hoppner et al. (1968).

This study was initiated to estimate the total vitamin A body stores and concentrations in human subjects. Subsequently, the report by Hoppner et al. was published from Canada in which it was stated that about 32% of the livers examined contained low stores of vitamin A (less than 40,0g/g). Consequently, the current study was expanded to determine whether U.S. populations have comparably low liver vitamin A concentrations. Human tissues were obtained for this study through the kind cooperation of Dr. J. Hood, University of lowa, and Dr. J. Greer, Ohio University.

RESULTS AND DISCUSSION OF THE RESULTS:

For analysis of vitamin A 1-2 grams of frozen stored wet tissue were used. Livers were ground with sodium sulfate and extracted with ether. All other tissues were soponified prior to ether extraction. Vitamin A was determined by the trifluor-oacetic acid procedure.

With the exception of liver, vitamin A showed no remarkable affinity for any of the 16 tissues examined regardless of liver concentration. Livers contained an average of 122, ug of vitamin A/g of liver and all other tissues contained about 1, ug/g tissue.

Examination of these same tissues showed that with the possible exception of adrenals all tissues had low and very variable concentrations of total carotenoids or β -carotene.
Based on the "average" man and the average tissue vitamin A concentrations it was estimated that the $122 \mu g$ of vitamin A/g of liver accounts for at least 85% of the total body stores of vitamin A.

In 9 livers which had levels of vitamin A from very low to very high it was found that the retinol fraction was relatively constant but the retinyl ester fraction increased with increasing vitamin A concentration.

When liver vitamin A concentrations were listed by arbitrarily chosen concentration levels (Table 1) it was found that the distributions were identical for the lowa and Ohio population groups. The age span of the subjects was 0 - 91 years of age with only four between the ages of 0 - 10 years of age. The median age in both groups was 50 years of age with a 2:1 male to female ratio.

Unlike the distribution of liver vitamin A concentrations the probable causes of death were somewhat different in the two groups. With the possible exception of the pulmonary disease group which contained the lowest liver concentration there did not appear to be any relationship between liver vitamin A concentration and probable cause of death, including accidental causes. These data do not agree with the older studies which showed that subjects that died of accidental causes had higher liver vitamin A concentrations than subjects whose death was disease related. The recent studies of Smith and Malthus did not find any relationship between cause of death and liver vitamin A concentration. Murray, reporting on the completed Canadian study (Vitamin A symposium, Boston, 1968), also stated that a cause of death relationship was not found.

CONCLUSIONS:

Liver vitamin A concentration in 88 human liver samples from lowa and Ohio contained an average of 122 µg/g liver with 29% containing less than 40 µg/g. Other tissues contained about 1µg/g tissue. Although these data are not adequate for an accurate evaluation of vitamin A nutriture they do point to the need for further studies particularly in view of their agreement with the Canadian findings that vitamin A insufficiency may be a greater problem in North America than was previously anticipated.

RECOMMENDATIONS:

These studies should be continued with other population groups with particular emphasis on cases of accidental deaths. Ideally, dietary histories should also

be available in order to determine whether the high incidence of low liver stores are due to an inadequate intake of vitamin A or to other causes.

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Studies in Human Nutrition (Cont'd)

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PUBLICATIONS:

1. Abstract.

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Raica, N., Jr., J. Scott, L. Lowry, H.E. Sauberlich and J. Hood. 1959. Vitamin A concentration in adult human tissues. Fed. Proc. 28, 190 (1989).

2. Manuscript providing detailed findings is in preparation.

STUDY NO. 2

Preparation of generally tabelled "C-beta-carotene for use in human vitamin A studies.

PROBLEM:

The human requirements of vitamin A and beta-carotene are being studied under a different protocol. This study involves the use of ¹⁴C labelled vitamin A and beta-carotene, the labeling of human subjects, the determination of vitamin A requirements, the pool sized and utilization rates of vitamin A, and the metabolism of the vitamin.

There is no commercially available source of C-beta-carotene. The only way to obtain uniformly labeled C-beta-carotene is to prepare it biosynphetically with a mold. This study reports the synthesis of uniformly labeled C-beta-carotene using the mold Phycomyces blokesleeanus.

RESULTS AND DISCUSSION OF THE RESULTS:

Initial attempts to prepare ¹⁴C-beta-carotene using P. blakesleeanus and slight modifications of the procedure of Lilly, et al. (Mycologia 50: 862, 1953) met with only partial success. Other attempts to prepare ¹⁴C-betacarotene using <u>Blakeslea</u> trispora and the methods of Purcell and Walter (J. Labelled Compounds **4** 94, 1968) produced an excellent yield of betacarotene but atter extensive purification, the radioactivity incorporated into beta-carotene fell to near zero.

The most promising organism for the biosynthesis of beta-carotene was P. blakesleggnus. Low level preliminary experiments were done to improve the yield of C incorporation into beta-carotene. Many variations in conditions were tried including growth of mated strains, inclusion of beta-ionone as a

stimulator of corptene synthesis, variation in glucose concentration, timing in the addition of 1°C, and changes in pH. The conditions reported belay are those that were found most effective in producing a maximum yield of 1°C incorporation into beta-corotene.

Phycomyces blakesleeanus, NRRL 1554, (-) strain was obtained from the Northern Regional Laboratory, U.S.D.A., Peoria, Illinois. The mold was maintained on a medium containing 5% malt, 2.5% agar and 0.8% yeast extract and subcultured at one month intervals. Spore suspensions were obtained by addition of sterile water to the maintenance of cultures followed by agitation and decapitation of the suspension into standard glucose-acetate medium.

Seed cultures were obtained by incubating a stirred, inoculated Erhlenmyer Flock for 24 hours at room temperature. The germinated spores were then used to inoculate four Fernbach flasks, each containing 900 ml of the medium. The Fernbach flasks were placed in a New Brunswick Psycotherm operated at 25°C with constant illumination, a shaking rate of 150 cycles per second, and a positive airflow through the gassing manifold. At intervals, the pH of the cultures was determined by removing a 2-3 this aliquot from one of the flasks. When the pth of the culture medium dropped to 3, (approximately 68 hours after innociulation of the seed cultures), the flasks were removed from the Psycotherm and the cells centrifuged at 1000 RPM for 5 minutes. The cells were resuspended in a medium identical to initial one except that it did not contain glucose or acehave, and the pH was 3.0. At this time, 10 mc of sodium acetate- $1, 2^{-1}$ C (specific activity = 54.0 mc/mmole) was distributed equally to the four Fernbach Flasks containing the resuspended cells. 2.5 mM of carrier acidified potassium acetate, pH 2.7-2.8 was added and the flasks put back in the Psycotherm. The Your flasks were connected to a 10% sodium hydroxide CO3 trap to trap respirwhory the CO3.

The cultures were incubated an additional 48 hours under conditions described previously. At 38 hour intervals, 2.5 mM of acidified potassium acetate was added to force the mold to grow on acetate. After a total incubation period of about 120 hours, the cultures were harvested. All operations from this point on were carried out in a darkened room.

After filtration of the cells and removal of the medium, the cells were extracted repeatedly with acetone using a blender. When the extract come through colortess, the combined acetone fractions were transferred to Petroleum ether (b.p. 30-80°) and saponified with an equal volume of 20% methanolic KOH for 30 minutes at slow' reflex. The non-saponifiable fraction was extracted with ipstroleum ether and washed free of alkali with water. The non-saponifiable traction was dried over antivdorus sodium sulfate for at least 30 minutes.

At each step in the purification, aliquots of the different fractions were rethrowed and counted in the scintillation counter. Samples containing wate, were counted in Brays solution. Colored samples in petroleum other were placed in empty vials and the petroleum ether blown off with a stream of introgen. 7.5 miles and the petroleum ether blown off with a stream of with an ultraviolet lamp with maximum output at 805 nm. 7.5 miles a corcentrated solution of phosphars was added so that the final mixture was identical in composition to the standard toluone-ethanol-PRO-dimental SORDE mixture. Automatic external standard toluone-ethanol-PRO-dimental powerhing.

The dy non-seponifiable fraction was quantitated at 450 nm using an extinction coefficient of E_{cm}^{bc} , 2500. After concentration of the sample to a small volume, the entire sample was applied to a 20 x 20 cm 1 mm thick have of silica get and developed with chloroform. In this system, the corotenes that the solvent front and the sterols, principally exposterol, trail behind. The sterol free carotenes were scraped off the plate, eluted and chromotographed on a 4.0 x 60.0 cm column of MgO; Hitle Super Cell (1:2 w w). The column was developed with suction using petroleum ether and mixtures of acetane-petroleum wither not exceeding 3% acetane. The beta-corotene band was removed and washed into petroleum ether and dryed over antrolous sodium sulfate. The spurified planent was crystallized with absolute ethanol - petroleum ether. Table 1 shows the data of one typical run.

The results show that although considerable radioactivity was taken up by the thold, that after several steps in the purification of beta-constene, and a small appoint of radioactivity was found in pure beta-constene. The sverall viela of the in pure beta-constene was 0.785%. This compares with a viela of 0.47% reported by Lilly, et al. on a preparation that was not as pure as this one. The specific activity obtained was 21.7 ypg/mg which compares with N.74% mg reported by Huang and Goodman (J. Biol. Chem. 240: 2839, 7465). The boteland of sufficient specific activity for use in human studies.

Two other runs were carried out with results similar to these. Attempts to improve the yield beyond that reported here were not successful. The location of the label in the purified pigment is general. This moons that every antition of the molecule has radioactivity in it, but that the specific activity at eact contain position may not be identical. Previous studies on the incorporation of acetate- C into bela-corrotene have shown that every contain position is inactual and that there is little randomization.

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Table !

Summary of the Results of a Typical Run on the Biosyntheis of Generally Labelled ¹⁴C-beta-carotene in <u>Phycomyces blakesleeanus</u>. Culture conditions are described in the text.

Fraction	Radioactivity (µC)	of β-carotene (μC/mg pigment)	^{I4} C Yield	
Acetate- ¹⁴ C	10,000	-	-	
Medium	812	-	-	
¹⁴ CO ₂ (as BaCO ₃)	3,200	-		
Acetone extract	2,150	-	2!.5	
Pet. ether extract	729	-	7.29	
Non-saponifiable	229	224.4	2.29	
Sterol-free carotenes	124	133,8	1.24	
β-Carotene (from MgO column)	18.5	21.1	0.185	
Rechromatographed β– carotene	18.1	22.0	0.181	

RECOMMENDATIONS: None

PUBLICATIONS: None

STUDY NO. 3

Experimental scurvy

PROBLEM:

The objectives of these studies were: (1) to measure by labeling with Lascorbic 1-¹⁴C, 4-³H acid, the body pool(s) of vitamin C and to study its rate of depletion during complete ascorbic acid deprivation, (2) to induce deficiency of ascorbic acid (scurvy) in healthy men, (3) to estimate the minimal requirements for ascorbic acid, (4) to observe the relationship between the size of the body pool(s) of ascorbic acid and clinical signs of scurvy, (5) to observe the effects of deficiency of ascorbic acid on certain physiological responses and, (6) to ascertain the amounts of ascorbic acid necessary to replete the body pool(s) and alleviate clinical signs and symptoms of scurvy.

RESULTS AND DISCUSSION OF THE RESULTS:

Recent observations of experimental scurvy^{1,2} confirmed (in one man) the previous studies by the British Medical Research Council (Special Report Series No. 280 H.M.S.O. London 1953) that the minimal amount of ascorbic acid needed to prevent or cure scurvy is slightly less than 10 mg, daily.

In that previously reported study the length of time required for the development of clinical signs and symptoms of mild scurvy in men fed a diet totally deficient in ascorbic acid was in the neighborhood of 90 days. Ascorbic acid depletion studies in man indicate that the plasma levels declined progressively until the 56th day of depletion after which time the test became invalid because of erratic results. Thin layer chromatographic (TLC) studies of serum at this time indicated that there were organic compounds present that reacted with dinetrophenylhydrazine (DNPH), yet were not L-ascorbic acid. Urinary excretion of ascorbic acid as measured by TLC methods reached zero levels before the 23rd day of depletion, however, the urine still contained unidentified substances which reacted with DNPH and was proven not to be Lascorbic acid.

Isotapic studies provided a preliminary estimate of the size of the total body pool as being approximately 1500 mg. Catabalism of the vitamin progressed at a constant rate of 1.55 per cent of the metabalically active pool. When this pool has been depleted to less than 300 mg, the arget of clinical scurvy occurred. The rate of catabalism of the dual ¹⁴C and ³H labeled ascorbic acid was the same. Repletion of the subjects with measured daily doses of radioactively labelled ascorbic acid demonstrated that a very small amount of this vitamin could ameliorate the signs and symptoms of scurvy. A daily dose of 6.5 mg appeared to be the absolute minimum for one subject. In all subjects the rate of repletion of the active body ascorbite pool proceeded in direct proportion to the daily dose. None of the vitamic was excreted in the urine either as reduced or oxidized ascorbic acid until the body pool had been restored to approximately 1500 mg. When the subjects were fed high intakes of ascorbic, only a limited quantity of the ingested vitamin was equilibrated with the labeled ascorbate pool. The remainder was excreted in the urine.

In the second sourvy study the degree of sourvy induced was more severe than it had been in the first. The clinical evidences of sourvy included not only hemorrhagic manifestations but also edema, severe arthralgias, a peripheral neuropathy in one man and the development of the Sicca syndrome in all subjects. Repletion of the men with varying doses of L-ascorbic acid msulted in complete recovery. In two subjects on this study 6.5 mg ascorbate/ day was not sufficient to completely relieve the signs of sourvy despite a prolonged repletion period.

CONCLUSIONS:

The results of the Sourvy II study are still being analyzed but appear to confirm and extend the results obtained from the Sourvy I study.

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STUDY NO. 4

Further studies on the vitamin B₆ requirement of yaung adult male humans,

PROBLEM:

In 1959, Harding, et al. reported that human volunteers subsisting for 24 days on a packaged military ration providing 165 grams of protein and 1.93 mg of vitamin B_6 per day developed a statistically significant elevation of xanthurenic acid excretion after a tryptophan load at the end of the period on the diet. The same subjects had no significant alteration in xanthurenic acid excretion post-tryptophan loading following 24 days on a puckaged ration providing 164 grams of protein and 2.76 mg of vitamin B_6 per day.

During subsequent years, a number of studies have been conducted at USAMRNL directed towards evaluating the human requirement for vitamin B_L. The studies have included experiments on the development of techniques for detecting an inadequacy of the vitamin as well as investigations on the influence of various dietary components on vitamin B_L requirement.

One dietary parameter that has been observed to influence vitamin B_6 requirement has been the daily dietary intake of protein. The requirement for the vitamin has been studied under controlled conditions in human volunteer subjects receiving a

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daily intake of protein ranging from 30 grams to 110 grams. At the higher level of protein intake, the daily requirement for vitamin B₆ appeared to be not in excess of 1.75 mg.

As was noted above, the initial study conducted with the packaged military ration provided a protein intake of 165 grams. In none of the subsequent studies was the vitamin B, requirement evaluated under conditions of a daily protein intake at this high level. Moreover, the subsequent studies have provided considerable experience that has led to improved techniques for evaluating the vitamin B6 requirements in mark.

In addition, further information was needed as to the storage stability of the nutrients, including vitamin B₆, provided in the presently employed "Meal, Combat, Individual" military ration (Erroneously referred to here and by many military personnel as the "C" ration). Thus, a study was desinged to provide additional information concerning the stability and availability of vitamin B₆ in the military "C ration" and the requirement for the vitamin at the high level of intake of protein provided by this ration.

RESULTS AND DISCUSSION OF THE RESULTS:

Eight volunteer research subjects on the Metabolic Ward were utilized for the study. The study was initiated on 6 January 1969 and continued through 31 March 1969. The subjects were placed on a cantrol diet for a period of seven days followed by 14 days on a vitamin B_0 deficient diet. During the remaining period of the study, the subjects received either "C-ration I" or "C-ration II". In the last phase of the study, the subjects received supplements of pyridoxine in accordance with each individual's urinary excretion of xanthurenic acid following a tryptophan load test. "C-ration I" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the standard "Meal, Combat, Individual" ration. "C-ration II" was the same ration, except that it had been stored for six months at a temperature of 99, 9 ± 0, 4°F and 57, 3 ± 0, 5% relative humidity prior to use in the study. During the investigation physical examinations and electroencephalographic measurements were performed. Urine and blood collections were made for use in evaluating changes that may occur in vitamin B, deficiency. Diets were made available for vitamin and approximate analyses. The data have been collected and are presently being evaluated.

CONCLUSIONS:

A study employing human young adult male subjects was conducted to evaluate the "Meai, Combat, Individual" rations in terms of their adequacy in vitamin B₆. The study was designed to provide information also concerning the stability of vitamin B₆ in the ration and an estimation of the human requirement for this vitamin when the daily dietary intake of protein is in excess of 150 grams. The data have been collected and are presently being evaluated.

?UBLICATIONS :

Canham, J.E., E.M. Baker, R.S. Harding, H.E. Sauberlich, and I.C. Plough. "Dietary Protein--Its relationship to vitamin Bg requirements and function. New York Academy of Science, In Press.

STUDY NO. 5

Other nutrition activities, national and international

PROBLEM:

Assistance and cooperation are provided to extend nutritional and medical research recommendations and training to U.S. military and civilian groups and to civil and inilitary populations of this and other countries as judged important and appropriate.

RESULTS AND DISCUSSION OF THE RESULTS :

Members of the Chemistry Division have assisted in the training program of the Reserve Officers' groups which during this past year involved participation in a nutrition survey of Ft. Irwin, California. Briefings and consultations in areas of nutrition were provided numerous visitors, including scientists from West Germany and Indonesia. Similarly, assistance has been provided in research, teaching, training and consulting to regional, national and international educationol or gavernment institutes. Included are such organizations as WHO and FAO of the United Nations, National Academy of Science - National Research Council, Univ. of California, Colorado State University, University of Colorado, University of Colorado Medical Center, University of Iowa Medical School, University of Denver, Oklahoma State University, National Institutes of Health, U.S. Public Health Service, etc. Of particular significance has been the assistance provided the U.S., Public Health Service in conducting nutritian surveys of populations in various states. The nutritional status of populations in Texas, Louisiana, Mississippi, Alabama, Arizone, Kentucky and New York have been investigated. Assistance was provided in a study conducted in Panama in which the Chemistry Division was to determine the electrolyte and water requirements of soldiers ingesting various diets while performing simulated cambat jungle duties. The data are under evaluation at present. An extensive study on the human requirement for vitamin A has been recently initiated an a cooperative basis with the University of Iowo Medical School. Professional assistance has been provided by staff members by serving as members of editorial boards of nutrition journals and as members of various scientific committees, including Project A.C.T.I.O.N. Advisory Committee, Nutrition Program Ad Hoc Advisory Committee of U.S. Public Health, HEW Sub-committee on Nutritian and Food Education, and FAO/WHO Expert Group on vitamins and mineral requirements.

CONCLUSIONS:

Continuing support was provided cooperating national and international nutrition agencies and their basic or applied nutrition programs and training activities.

PUBLICATIONS:

1. Lowry, L.K., Y.F. Herman, and H.E. Sauberlich. "Preparation of Riboflavin (UL)- ${}^{14}C$." (Submitted to MR & D for clearance prior to submission for publication.)

2. "Recommended Dietary Allowances", Seventh Edition, 1968, National Academy of Sciences.

3. Ziporin, Z.Z. and P.P. Waring. Thin-layer chromatography for the separation of thiamin, N'-meiliylnicotinamide and related compounds. To be published in Colowich, S.P. and N.O. Kaplan, "Methods in Enzymology," volume on "Vitamins and Coenzymes."

4. Ziporin, Z.Z. and P.P. Waring. The use of yeast to assay the separate moieties of thiamin. To be published in Colowich, S.P. and N.O. Kaplan, "Methods in Enzymology", volume on "Vitamins and Coenzymes."

5. Ariaey-Nejad, M.R., M. Balaghi, E.M. Baker, and H.E. Sauberlich. Studies on thiamine metabolism in man (In press, Am.J. Clin. Nutrition).

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ABSTRACT

PROJECT NO.	3A062110A822	Military Internal Medicine
WORK UNIT NO.	073	Applied Nutrition Studies of Military Populations

STUDY NO.

Nutrition surveys in military installations were continued in 1968 to determine the nutrient intake and the nutritional status of military personnel under various climatic conditions. The fifth in this series of surveys was conducted at Fort Irwin, California, in a hot, dry desert environment. Previous surveys were conducted at Fort Carson, Colorado; Fort Benning, Georgia; Fort Huachuca, Arizona, and Fort Campbell, Kentucky. The Fort Irwin survey also served as a training mission for the mobilization designees (reserve officers) assigned to USAMRNL.

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STUDY NO.

Four studies have been completed to determine the minimal food intake necessary to permit the individual soldier to effectively perform his duties for periods of 3, 7 and 10 days. These studies included 10 days of complete starvation, two studies of caloric restriction (420 and 500 Cal/day) for 10 days, and a field study in the jungles of Panama. Four groups of men (Panama Study) received 600, 1000, 1500, and 3600 Cal/day, respectively. In the 420 Cal/day study, the major adverse effects were quite similar to the starvation study and included abnormal EEG tracings and negative nitrogen balances in the nonmineral supplemented group, and negative nitrogen balances in the mineral supplemented group.

The inclusion of protein in the 500 Calorie intake per day (with 3600 Calorie expenditure) did not significantly improve nitrogen balance in comparison to the 420 Cal. of carbohydrate intake and 3200 Cal. expenditure. Urinary ketones and greater water losses were observed in the 500 Cal. study compared to the 420 Cal. study, which would indicate that 85 grams of carbohydrate per day was insufficient under these conditions to prevent ketosis. Mineral supplementation in one half of the subjects of both the caloric restriction studies, conserved body water. The biological samples from the Panama Study have not been analyzed.

BODY OF REPORT

WORK UNIT NO. 073

Applied Nutrition Studies of Military Populations

STUDY NO.

Nutrition Surveys

PROBLEM:

Annual Army post nutrition surveys will be conducted to evaluate the adequacy of the Army diet in terms of established recommended allowances under varied climatic conditions. Diet analysis will also include the essential nutrients for which recommended dietary allowances have not been established. Clinical evaluation of the nutritional and physical status of military personnel is essential in addition to the biochemical evaluations of the blood and urine samples. A special effort will be made to evaluate body composition, work performance, and cardiopulmonary measurements in terms of dietary intake, habits and nutritional status.

RESULTS AND DISCUSSION OF THE RESULTS:

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Data from the survey at Fort Irwin, dealing with food consumption of the three separate groups of individuals, are currently being evaluated; results will appear shortly as a laboratory report. This survey also served as a training mission for the mobilization designees (reserve officers) assigned to USAMANL.

Progress has been made in compilation of extensive data from earlier surveys. In collaboration with the Computer Division, USAMRNL, analysis by computer is being carried out on 44 parameters relating to body composition, work performance and respiratory function which were gathered during the Fort Carson, Fort Huachuca, and Fort Campbell surveys. Information on eight age groups have been punched on tape and are being analyzed for means, standard deviations, and correlation coefficients. These data should also be available shortly for publication. A paper entitled, "Nutrient Intake and Nutritional Status of Selected Military Populations", was presented at a symposium entitled, "Nutrition U.S. A.", at the 1969 FASEB annual meeting. Finally, this Division has participated in the National Nutrition Survey conducted by the U.S. Public Health Service in 1968-1969 on Navajo Indians at Lower Greasewood, Arizona.

Applied Nutrition Studies of Military Populations (Con't)

CONCLUSIONS:

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The primary purpose of these studies is to conduct nutrition surveys on a representative sample of U.S. Army personnel and to provide information necessary for execution of AMEDS responsibilities under AR 30-11 and AR 40-25. The Fort Irwin, California study was the fifth in the current series of surveys. These studies have been directed to extend for an additional five year period.

RECOMMENDATIONS:

Future studies will include additional surveys in basic training camps for both men and women and the evaluation of the blood lipid fractions and vitamin A status of military populations consuming reconstituted milk for periods of up to one year.

PODY OF REPORT

WORK UNIT NO. 078

Applied Nutrition. Studies a Military Populations

STUDY NO.

Calling Restruction.

PROFLEMS:

Recent emphasis on the mobility of our military strees inner condtions where food resupply is difficult, have created new problems is providing sufficient food and whiter for symbolic personnel to maintain adequate performance. The soldier in combat situations for periods up to 40 days musi corr, his pack, radio equipment, weapons, and as adequate supply of food and water which is usually quite norm, and bulky. The military has been concerned about the minimal food utake necessary to effectively maintain physical effecters a for survinperiods where resupply is impossible. As a result, a series a studies have been initiated to determine the minimal colors and mutrient requirement to maintain effectively performance of the indicatual for periods up to 10 days.

RESULTS AND DESCUSSION OF THE RESULTS:

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Two studies were published on caloric restriction using 420 Cal (da, (2, 3). Some adverse effects were still observed and are summarized. Eight healthy young adults consumed 420 Cal/day for the 10-day period; one group (1) with mineral supplements, and the other (11) with no supplementation. Nitrogen balances were not as negative as during 10-days of complete starvation; however, these nitrogen losses were still greet indicating that the effect of limited carbohydrate ingestion, upon reducing protein carbohism was minimal. The men in Group II all had abnormal EEG tracings during restriction while the EEG's of then in Group I remained normal. Group II still showed some fairly large body water losses thiring Days 1 and 2 of restriction wheel was rot apparent in Group I.

Analysis of data from the 1900 study or, the metabolic effects at 7.500 Cal/day diet, composed of carboliedrates and proteins with or without mineral supplements has been completed. Metabolic findings of this study were rather similar to those obtained following restriction of diet to 420-Cal. of carboliedrate (day with or without mineral supplements.

Applied Nutrition Studies of Military Populations (Con't)

A field study on caloric restriction was conducted in September and October 1968 in the Panama jungle. Six groups of 20 men each participated in maneuvers in the jungle environment. Each group received different diets, which included 600, 1000, and 1500 Cal/day for 10 days; Group 4 received the C-ration (MT), another consumed the dehydrated Long Range Patrol ration, and the last group ate a regular military dist of approximately 3600 Cal/day. This study was designed specifically to gather information on the minimal nutrient requirements of troops during limited simulated combat patrols. Samples of the six types of diets, urine and sweat were collected in order to estimate mineral and nitrogen balances during intake of various diets (lowest 600 Cal/day) in a tropical environment. These samples will be analyzed following completion of the previously mentioned high altitude study. Other data on food intake, nutrient balances, work performance, body composition, and respiratory function are being processed at the present time.

CONCLUSIONS:

In the 420 Cal/Carbohydrate/day study for 10 days, the daily energy expenditure was 3200 Cal/day, and it was observed that limited calories without mineral supplementation appeared to be more beneficial than complete starvation. Some major abnormalities were still present including fairly large body water losses, significant protein losses and abnormal EEG tracings in all men from this group. Mineral supplementation with limited calories had beneficial effects in reducing water deficits and hypohydration, preventing ketosis and in preventing abnormal EEG tracings.

Mineral metabolism studies have been completed and manuscripts prepared on the 500 Calorie study.

RECOMMENDATIONS:

The completion of the jungle study in Panama will provide an opportunity to evaluate the minimal food requirements for simulated combat patrols for periods up to 10 days.

Applied Nutrition Studies of Military Populations (Con't)

PUBLICATIONS:

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ABSTRACT

PROJECT NO.	3A062110A822	Military Internal Medicine
WORK UNIT NO.	074	Nutritional and Metabolic Aspects of Nutrients

The following investigations have been conducted under this work unit:

- STUDY NO. 1 Utilization of β-carotene in the vitamin A deficient germfree rat.
- STUDY NO. 2 Nutritional and Wholesomeness aspects of irradiated foods.
- STUDY NO. 3 Metabolism of nicotinic acid and nicotinamide in gnotobiotic rats.
- STUDY NO. 4 Malabsorption, diarrhea, steatorrhea and nutritional deficiency syndromes.
- STUDY NO. 5 Tissue and plood enzymes.
- STUDY NO. 6 Effect of a nickel-low, arginine-high diet on chicks.

1. Of the two reports on vitamin A deficiency in germfree rats one stated that the time-course of deficiency and death was comparable in germfree and conventional rats, the other stated that the deficient germfree rat's longevity was greatly increased. There are no reports on the biological efficiency of β -carotene in the germfree rat. This study confirms that vitamin A deficient germfree rats not only develop the classical vitamin A deficiency lesions but have increased longevity in the germfree state and can utilize β -carotene for growth and liver storage as effectively as conventional rats.

2. Irradiation preservation of foods is still of interest to the Army and requires continued liaison and assistance to USAMRDC in regard to wholesomeness. Most of the efforts during this past year have been concerned with the preparation of statements and appearances before the Congressional Subcommittee on Research, Development and Radiation as well as assisting in the preparation of a new feeding protocol.

3. Studies on the metabolism of nicotinic acid and nicotinamide in gnotobiotic rats have been completed and the results are currently in press.

4. To explore the possibility that an infectious or toxic agent is involved in the tropical sprue syndrome lyophilized stool samples or cell free filtrates (0.45 micron) of stool homogenates from tropical sprue patients were fed to germfree rats. After eight weeks there were no untoward symptoms to suggest a sprue syndrome. Histopathologic examination of the intestines showed normal mucosal structure.

5. The effect of intestinal microflora upon zinc metabolism was studied using germfree (GF) and pathogen-free (PF) rats. Isotope levels in the urine and feces were monitored following oral administration of 65 Zn. Fecal excretion of the isotope did not differ significantly between GF and PF rats; urinary excretion of 65 Zn, even though relatively low in both groups, was significantly greater in the GF rats. There were no significant differences between the two groups in: (a) the amount of 65 Zn remaining in the gastrointestinal tract 48 hours following oral administration, (b) the amount of 65 Zn in the cecum, nor (c) the amount of 65 Zn deposited in the liver and kidneys during the 48 hours following dosage. Feed consumption (or efficiency) and growth rates were comparable for GF and PF rats.

6. During a study of the effect of a folate deficient diet on jejunal glycolytic enzymes in germfree (GF) and pathogen-free (PF) male rots the pentose phosphate pathway (PPP) of the red blood cells (RBCs) obtained from these animals was also studied. The production of ${}^{14}\text{CO}_2$ from 1– ${}^{14}\text{C}$ -glucose was used as an index of R3C PPP metabolism. The response of intact and hemolyzed R3Cs to riboflavin, thiamine and folic acid was measured. GF and PF rats were placed on a folic acid deficient diet for 24 days and then bled at 0, 6, 24, and 72 hours. The rats bled at 6, 24 and 72 hours were given 150 µg of folic acid orally, daily. Control GF rats, on a folic ocid complete diet, were treated similarly. PF rat R3Cs were obtained at zero time only. No anemio or megaloblastosis was seen. At zero time, when jejunal alycolytic enzymes of folic acid deficient rats were significantly decreased, the PPP of intact GF rat RBCs did not stimulate with 150 µg of added folic acid, while the PPP of intact PF rat RBCs did stimulate. Hemolysates of both GF and PF rat RBCs had increased activity of the PPP with added folic acid. The intact GF RBCs obtained at 6, 24 and 72 hours did not stimulate with added folic acid and appeared to be quite resistant to dietary folute deficiency even though the jejunal glycolytic enzymes were quite sensitive to folate deficiency and repletion.

7. Studies were conducted to determine whether or not nickel plays a physiological role in the chick. Day-old White Rock chicks, kept in a metal-free environment, were fed a corn meal-skim milk powder diet which contained <0.08 ppm nickel on an air dried basis. Control chicks were kept under the same conditions, but

had an additional 5 ppm nickel supplemented to the diet. Difference in the uptake and distribution of ⁶³Ni in chicks on these 2 dietary regimes were also studied. Feeding i ickel-low diets to chicks resulted in gross changes which were accentuated by a high arginine level (74%) in the diet. Differences noted were: (1) a change in pigmentation in the legs, and to a lesser extent, the beak; and (2) legs with slightly swollen hocks and thicker bones. The length: width ratio of the tibias significantly decreased in the chicks fed the nickel-low diets when compared to those fed the supplemental nickel. No differences in weights were noted. Because of the variability in results and small number of degrees of freedom, few significant changes were noted in the isotope study. The chicks fed the nickellow diet retained a significantly greater amount of ⁶³Ni at the 6 hour time period after administration of isotope in the bone, aorta, and liver in experiment 2 (high arginine diets). From the results, it appears that nickel has a physiological role in the chick.

BODY OF REPORT

WORK UNIT NO. 074

Nutritional and Metabolic Aspects of Nutrients

STUDY NO. 1

Utilization of β -carotene in the vitamin A deficient germ-free rat.

PROBLEM:

The characteristics and vitamin A requirements of germfree (GF) rats, unlike the conventional rats, have not been adequately studied. Only two reports have appeared on vitamin A deficiency in GF rats. Beaver (1961) reported that although the time-rate of onset of deficiency and death in the GF rat was comparable to the conventional rat, lesions in soft tissues were more extensive in the GF rat. Bieri et al. (1969) reported that the vitamin A deficient GF rat survived for many weeks after the onset of growth retardation.

This study was initiated to study the effectiveness of β -carotene in GF rats for growth and liver storage of vitamin A.

METHOD:

Mature GF female and male rats were purchased from Charles River Breeding Labs, and bred in a sterile environment. During lactation the mothers were fed a steam sterilized 22% case in laboratory diet deficient in vitamin A. The composition of this diet except for added vitamin A was identical to that normally fed to GF rats in this laboratory.

Young were weared at about 21 days and were continued on the vitamin A deficient diet for 148 days, at which time the remaining rats (4 males and 6 females) were divided into two equal groups for supplementation. Daily supplements of 15 μ g of retinol or 28 μ g of β -carotene in cottonseed oil were administered orally for 19 days. The oil solutions were sterilized by filtration. The rats were sacrificed after a 24 hour fast. Tissues were removed for pathologic examination and measurement of liver retinol stores. Livers were saponified, extracted with ether and retinol determined by the trifluoroacetic acid procedure.

RESULTS AND DISCUSSION OF THE RESULTS:

The growth of the rats plateaued after 80 days on vitamin A deficient diet. After 140 days two males that had become lethargic because of cecal volvulus were sacrificed. Liver retinol was found to be less than 0.5 µg/liver. One

female who had received no supplementation throughout the entire experimental partial was found to have after 169 days on deficient diet a comparable liver retinul content. Liver stores of two male rats which were sacrificed because of volvulus after 6 and 13 days of supplementation with retinal were 9.6 μg and 16.2 ug/liver, respectively. The two male rats which were supplemented with β-carotene died before they could be sacrificed.

Weight gains and liver retinol data for the supplemented rats at the termination of the study are shown in Table 1. Weight gains are equal in the two groups. Although the liver vitamin A stores in the β -carotene group are slightly lower the data suggest that the biological equivalency of retinol and β -carotene are comparable in GF and conventional rats. That is, 0.3 µg of retinol is equivalent to 0.6 µg of β -carotene for growth and liver storage of vitamin A.

TABLE I

WEIGHT

Weight gains and total liver vitamin A stores of germfree rats following repletion.¹

	Start	End	Retinol
	<u>(g)</u>	(g)	(ug/liver)
Catotene	174 ± 4	195 ± 3	70.8±1.4
Retinol	166 ± 9	185 ± 14	85.3 ± 11.3

¹Three females per group. Supplemented daily with 28 μ g of β -carotene or 15 μ g of retinol for 19 days.

On necropsy all animals had worn, chalky, white teeth. Males had enlarged accessory sex glands and flaky proteinaceous material in the urinary bladder. Eye lesions were absent in the animals examined grossly or microscopically.

Odontopathy of the incisor teeth was consistently present and was quite characteristic of vitamin A deficiency. Squamous metaplasia with cornification was seen in several other tissues. Kidneys contained calcium crystals. Sperm production was not evident in either male rat examined.

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Nutritional and Metabolic Aspects of Nutrients (Cont⁴d)

Moore (1957) in his review cited data to suggest that vitamin A may be required for detoxification. Bieri et al. (1969) and other have suggested that conventional vitamin A deficient rats succumb to infection within a few days after growth plateau. It would seem, therefore, that the vitamin A deficient GF rat is protected not only from infection but also from microbial toxicants. Hence, the GF rat may have a reduced vitamin A requirement and increased longevity in the vitamin A deficient state.

CONCLUSION:

The biological effectiveness of retinol and β -carotene are comparable in GF and conventional rats as measured by growth and liver retinol stores. Vitamin A deficiency was established by low liver stores of retinol, growth arrest, mild hind quarter paralysis, transient diarrhea, response to retinol or β -carotene and pathologic data. The increased longevity of vitamin A deficient GF rats has been confirmed.

RECOMMENDATIONS:

The increased longevity of vitamin A deficient GF rats in spite of the development of classical lesions suggest the re-examination of the discarded concept that vitamin A is an "anti-infection" vitamin.

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PUBLICATION:

Manuscript submitted for publication in Proceedings of the Henry Steenbock Symposium on the Fat Soluble Vitamins, Madison, Wisconsin, June, 1969.

Raica, N., M.A. Stedham, Y.F. Herman and H.E. Sauberlich. Vitamin A. deficiency in germfree rats.

STUDY NO. 2

Nutritional and Wholesomeriess aspects of irradiated foods.

PROBLEM:

This laboratory has continued to provide technicol support to Medical R & D in areas on the wholesomeness of irradiated foods. With the denial of the Army ham petition and cancellation of the previous approval of irradiated bacon by the Food and Drug Administration (FDA) the Army must reinitiate long-term wholesomeness feeding studies if irradiation starilized meat items are derived by the Army.

RESULTS AND DISCUSSION OF THE RESULTS:

Efforts during this past year have been placed primarily on assisting Medical R & D in the writing of a new protocol for the wholesomeness testing of irradiated food.

The recessity for a new protocol has resulted from FDA's adverse reaction to existing wholesomeness data. The new protocol, which is presently being reviewed by FDA, is designed to answer specific questions raised by FDA as well as to update the approach and thinking incorporated in the protocols written prior to 1959.

Work meetings were attended in Washington and Natick Laboratories in regard to the hearings before the congressional Subcommittee on Research, Development and Radiation and the new protocol.

Reports of past studies on wholesomeness are still being sent upon proper requests to foreign countries and their representatives.

A seminar on Toxicity Testing of Foods was presented at Natick Laboratories. 22 November, 1968.

CONCLUSION:

The developments during this past year clearly indicate that if irradiation starilized foods are desired by the Army or any other agency new feeding studies and tests which conform to current attitudes must be completed. However, in spite of FDA's adverse reaction to irradiated foods the worldwide interest in irradiated foods continues to be marked.

RECOMMENDATIONS: None

PUBLICATIONS: Nona

STUDY NO. 3

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Metabolism of nicotinic acid and nicotinamide in gnotobiotic rats.

This study has been terminated and all work is currently in press.

PUBLICATIONS:

Abstract. Lee, Y.C., R.K. Gholson and N. Raica. Identification of two new metabolites of nicotinamide in germfree rats. American Chemistry Society, Division of Biological Chemistry, Proceedings, Sept. 9 – 13, 1968. Abstract No. 24.

Lee, Y.C., R.K. Gholson and N. Raica. Isolation and identification of two nicotinamide metabolites. J. Biol. Chem., In Press.

STUDY NO. 4

Malabsorption, diarrhea, steatorrhea and nutritional deficiency syndromes.

PROBLEM:

The presence of "normal" intestinal microflora, pathogenic and nonpathogenic complicates the study of nutrient absorption and utilization in the normal or diseased animals. The objectives of this study are to study the absorption and utilization of nutrients in the gnotobiotic and conventional rat and to determine the effect of nutritional deficiencies, toxic food components and/or microorganisms on the histology of the intestinal mucosa and malabsorption syndromes. One approach will be an attempt to induce a tropical sprue syndrome in germfree rats by inoculation with crude fecal homogenates or cell-free fecal filtrates from patients with tropical sprue. Other studies will, utilize radio-labelled nutrients and vitamin deficiencies to determine absorption and utilization in the normal and diseased germfree animal. The following experiments have been conducted:

RESULTS AND DISCUSSION OF THE RESULTS:

(a) Attempts to induce a tropical sprue syndrome in germfree rats. Young germfree rats were fed lyophilized stool samples (2 grams in 50 grams of diet

plus 5 grams vagetable oil) from tropical sprue patients. Homogenates in horse serum were also made from the frozen stools and filtered through a sterile 0.45 micron filter. One ml. of the filtrate was administered orally on a daily basis to another group of germfree rats. After 8 weeks neither group of anima's showed any untoward symptoms or evidence of a sprue syndrome. Histopathological examination of the intestinal mucosa did not reveal any abnormality.

CONCLUSIONS:

Under the conditions of this study it would appear that the stool samples from the available tropical sprue patients did not contain a unique infectious or toxic agents that could induce a malabsorption syndrome in the germfree rat. Results of this study are inconclusive. Until stool samples and upper gastric washings from untreated tropical sprue subjects from areas in which antibiotics are effective in "curing" the sprue are available the above inoculation approach should not be continued.

PUBLICATIONS: None

(b) Absorption and metabolism of essential minerals in the germfree animal.

The use of germfree animals for basic metabolic studies poses some rather elementary questions concerning the nutritional adequacy of diets fed the control animals. If the intestinal microflora affect metabolism, then one must examine the effect of this microflora upon the availability and absorption of the various nutrients. Only then will it be possible to develop a truly adequate diet for germfree control animals. The study to be reported was to determine whether or not zinc metabolism is influenced by intestinal microflora.

Previous reports (MRNL Progress Report June 1968) indicated that copper nutrition may not be the same in germfree (GF) and pathogen-free (PF) rats. Continuing the study of the effect of intestinal microflora upon mineral nutrition, zinc metabolism was studied in the experiments to be reported here. Eight GF and eight PF male, weanling rats of the Charles River CDF strain were used. The GF animals were housed in individual, stainless steel, wirefloor cages inside a sterile, plastic chamber; and the PF rats were kept in individual, stainless steel, wire-floor cages in an open colony room. All animals received the same steam-sterilized, casein-com starch diet and water ad libitum. At the end of a three week period, during which growth rate and feed consumption data were recorded, each animal was intubated with an aqueous solution containing 20 μ Ci of ⁶⁵Zn. The rats were equipped with tail cups and placed in metabolism cages where feed and water were provided ad libitum. Urins and feces were collected for 48 hours and radioactivity determinations made on all collected samples.

At the end of the collection period, the rats were sacrificed and the livers and kidneys were removed for isotope determination. After proper ligations to prevent fluid loses, the gastrointestinal (GI) tract (including the stomach, and small and large intestine) and the cecum were removed from each animal, also for radioactivity determinations.

The effect of environmental status upon ⁶⁵Zn excretion, growth rates, and feed consumption is summarized in Table 1. During the 48 hour period following oral administration of ⁶⁵Zn, there was no significant difference between the GF and PF rats in the amount of isotope excreted in the feces. This observation would seem to indicate that the intestinal microflora had little, or no, effect upon zinc absorption. Despite the very low activity found, the GF rats excreted significantly more ⁶⁵Zn in the urine than their conventional counterparts. This observation might suggest that GF rats have a more rapid rate of zinc turnover than do PF rats. Growth rates and feed consumption were similar for the GF and PF rats, indicating that no overt nor gross differences in nutrient utilization exist between animals in the two environments.

TABLE I

Effect of Environmental Status on ⁶⁵Zn Excretion, Growth Rate and Feed Consumption

	65 Zn Excretion					
Environment	Urine	Feces	Growth Rate	Feed Consumption		
	(% of oral dose)		(g∕day)	(g∕g gain in body weight)		
Germfree	1.04	48.0	3.50	2.44		
Pathogen-free	0.60	44.0	3.90	2.73		

The effect of germfree conditions upon tissue distribution of ⁶⁵Zn is summarized in Table II. There were no significant differences between GF and PF rats in the residual radioactivity in the GI tracts and the ceca, again supporting the suggestion that intestinal microflora have very little, if any, effect upon zinc absorption. Similarly, the amount of ⁶⁵Zn retained by the livers and kidneys was not significantly different between the GF and PF animals. Thus, the

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tissue data and the urinary data seem to be contradictory; the former suggesting no difference in zinc turnover between GF and PF rats; but the latter indicating a more rapid zinc turnover rate for GF rats. No explanation for this apparent voriance is yet available.

TABLE II

Effect of Environmental Status on Tissue Distribution of ⁶⁵Zn.

Environment							
	GI ^a	Liver	Kidneys	Cecum			
	(% oral dose)						
Germfree	10.9	6.54	1.77	22.2			
Pathogen-free	9.24	7.36	2.20	17.9			

^aGastrointestinal tract including stomach, small intestine, and large intestine.

CONCLUSIONS:

Contrary to the effect of germfree conditions on copper nutrition, germfree and conventional rats appear to differ little, if any, in their general zinc nutrition. However, the present data cannot be construed to mean that more subtle characteristics of zinc metabolism than those measured might not differ between GF and PF rats.

RECOMMENDATIONS:

Continue to use germfree animals to study the effect of intestinal microflora on mineral metabolism. More refined and detailed experimentation should be used in order to study the possible subtle differences which might be overlooked by the presently used general experiments.

PUBLICATIONS:

Dowdy, R. P., Y.F. Herman, and H.E. Sauberlich. Effect of germfree status on ⁴Cu excretion by the rat. Proc. Soc. Exptl. Biol. Med. <u>130</u>: 1294 – 1297, 1969.

STUDY NO. 5

Tissue and blood enzymes

PROBLEM:

To study the mechanism of action of various vitamins which affect the pentose phosphate pathway of animal red blood cells, particularly gnotobiotic rats on a folic acid deficient diet, and to determine whether the red blood cell pentose phosphate pathway is affected by the folic acid deficient diet in the same way as the jejunal glycolytic enzymes.

RESULTS AND DISCUSSION OF THE RESULTS:

Studies in the Metabolic Division showed that dietary sugars increased the activities of the specific enzymes of the jejunum involved in their metabolism in both man and rat. Thus, dietary fructose increased the activities of jejunal fructokinase, fructose-1-phosphate aldolase, fructose-1,6-diphosphate aldolase and pyruvate kinase. Dietary glucose increased the activities of jejunal glucokinase, hexokinase, fructose-1,6-diphosphate aldolase and pyruvate kinase. These studies led to collaboration between the Metabolic and Chemistry Divisions to investigate the effect of oral folic acid on the glycolytic enzymes in man (1). We then investigated the effect of oral folic acid on the jejunal glycolytic enzymes of male germfree (GF) and male pathogen-free (PF) rats; germfree animals being chosen particularly because of the reported difficulty in demonstrating folic acid deficiency without administering an antibiotic. The results of these studies showed that the GF rat on a folic acid deficient diet had a decreased serum folate level and a marked decrease in the jejunal alycolytic enzyme activities which increased when oral folate was administered. It was during the course of these studies that rat red blood cells (RBC's) were obtained from GF and PF male rats, strain CDF, of the Charles River Breeding Laboratories, Wilmington, Massachusetts, and the pentose phosphate pathway (PPP) was studied in the RBC's obtained from folic acid deficient and folic acid replete rats.

The production of ¹⁴C-labelled carbon dioxids (¹⁴CO₂) from the first carbon of ¹⁴C-labelled glucose (1-¹⁴C-glucose) was used as an index of R3C PPP metabolism. Measurements were obtained by trapping the ¹⁴CO₂ in hyamine hydroxide and counting this activity in a liquid scintillation counter. The results were calculated as the mean percent of the initial radioactivity recovered as ¹⁴CO₂. The production of ¹⁴CO₂ was measured in both the intact R3C's and RBC hemolysates with or without the following cofactors: Thiamine (B₁), 2 mM; riboflavin (B₂), 30µg; and folic acid, 150 ug added

to each incubation flask. Twenty-four GF rots weighing 40 grams each were placed on a folic acid complete diet (+FA) for 7 days, at the end of which 16 of the 24 GF rats were placed on a folic acid deficient (-FA) diet and the remaining 8 GF rats were continued on the +FA diet. Both groups of GF rats were maintained on this dietary regimen for 24 more days. At the end of the 24th day the study was begun when at zero time 4 GF rots on the -FA diet (experimental) and 2 GF rats on the +FA diet (Control) were sacrificed. The remaining GF rats in both groups (experimentol and control) were given 150µg of folic acid by stomach tube at 0, 24 and 48 hours. Four of the GF rats on the -FA diet (experimental group) ond 2 GF rats on the +FA diet (control group) were sacrificed at 6, 24, 72 hours, respectively. Animals were weighed prior to death, anesthetized with ether, bled by cordiac puncture, and the blood placed into heparinized tubes. Hematocrits were done by the microhematocrit technique and blood smears also were made.

R3C suspensions were prepared by removing the plasma by centrifugation, washing the cells 3 times in Krebs-Ringer-phosphate buffer, pH 7.4, discarding the buffy coat and top layer of the washed packed R3C's, and resuspending the washed packed RBC's with buffer in a 1:1 proportion. An amount of 0.2 ml of cells from a pool of blood from several animals was used in each incubation flask. Each flask contained 22, 200 dpm of $1-{}^{14}C$ -glucose (S.A. 1.79 millicurie/mmole) in Krebs-Ringer- phosphate, pH 7.4 with o total volume of 3.0 ml. Hemolysates were prepared by substituting water for the buffer. Cofactors were added in the amounts previously indicated. Red blood cells from PF rats were obtained at zero time only but otherwise treated the same.

Table I summarizes the results of intact RBC's and R3C hemolysate obtained from GF rats on -FA and +FA diets. The production of $^{14}CO_2$ at zero time and at 6, 24 and 72 hours after the start of folic acid repletion is represented as the mean percent of the initial radioactivity with and without the added thiamine, riboflavin and folic acid. At zero time the experimental (-FA) intoct RBC's and its control (+FA) intact RBC's did not stimulate with any of the added cofactors including folic acid, though the level of activity of the control (+FA) intact RBC's was somewhat higher than that for the experimental (-FA) intact RBC's.

TABLE I

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The production of ${}^{14}CO_2$ from $1 - {}^{14}C$ glucose by intact and hemolyzed red blood cells of germfree rats depleted and repleted of folic acid.

		Intact	RBC's	RBC Hamolysote		
	additions	GF (-FA)	GF (+FA)	GF (-ፑA)	GF (+FA)	
	none	11.1	17.3	20.0	30.2	
Zelo	в,	14.1	18.2	15.3	20.3	
time	B ₂	12.5	18.5	40.3	43.8	
	FĂ	12.1	18.1	36.0	43.6	
6 hrs.	none	12.5	12.3	18.3	10.2	
after	B	13.2	12.7	23.9	23.6	
Intubation	B ₂	13.2	12.7	40.1	42.5	
with FA	FA	13.4	14.2	33.4	16.2	
24 hrs.	none	8.8	13.4	5.9	7.6	
after	B	8.6	13.2	12.2	33.5	
intubation	B ₂	9.3	13.7	42.3	37.9	
with FA	FA	10.6	14.2	6.2	37.7	
72 his.	none	17.2	15.8	19.0	16.1	
after	6,	17.6	16.9	34.3	25.5	
intubation	ь. Б2	17.1	15.9	51.5	53.5	
with FA	FÁ	20.4	19.1	33.6	27.2	

Values are given as the percent of initial radioactivity recovered as ${}^{14}CO_2$. The initial amount of 1- ${}^{14}C$ -glucose used was 22, 200 dpm. 150, ug folic acid added to each flask.

A volume of 0.2 ml. of a 1:1 diluted packed red blood cells was used per flask.

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At 6, 24 and 72 hours the intact GF RBC's, both -FA and +FA, did not stimulate with any of the added cofactors, including folic acid though the latter at 72 hours did stimulate slightly more than without the cofactors. The intact GF RBC's appeared to be quite resistant to dietary folate deficiency even though jejunal glycolytic enzymes were quite sensitive to folate deficiency and repletion.

Results with and without the various cofactors at zero time were very similar for the RBC hemolysates obtained from both experimental (-FA) and its control (+FA) GF rats. At 6 hours after folic acid repletion the RBC hemolysate from the -FA rats still responded to added folic acid; while the +FA GF rat RBC hemolysate did not. At this point thiamine increased the PPP octivity in hemolysates from both groups. At 24 hours the RBC hemolysates without cofactors, both -FA and +FA, had decreased PPP activity; however, at this time the RBC hemolysates from the experimental group (-FA) responded poorly to thiamine and folic acid though still responding to riboflavin; whereas control (+FA) RBC hemolysate responded to all 3 cofactors. At 72 hours the experimental (-FA) RBC hemolysate lost most of the capability to respond to thiamine and folic acid.

Table II compares the results obtained at zero time for the GF and PF rat RBC's, both intact and hemolysate, with and without folic acid addition. When the activity of jejunal enzymes of folate deficient rats was significantly decreased at this time and the plasma folate level was significantly lower in rats on a folate deficient diet as compared to the folate complete, the PPP of the intoct RBC's from both -FA and +FA GF rats was not stimulated with 150 µg of added folic acid; while the intact RBC's from -FA and +FA PF rats were stimulated. Hemolysates of GF and PF rat RBC's had increased PPP activity with added folic acid, with the greater increase seen in RBC hemolysates from both GF and PF rats on the folic acid deficient diet. No unemia or megaloblastosis was seen in these rats.
TABLE II

Comparison of the 1-¹⁴C-Glucose Metabolism Between Intact and Hemolyzed Red Blood Cells of Germfree and Pathogen-free Rats on a Folic Acid Deficient Diet.

		Intact F	IBC's	RBC Hem	olysate
-FA Diet	additions none FA	GF 11.1 12.1	PF 11.9 31.2	GF 20.0 36.0	PF 10.4 38.1
+FA Diet	none FA	17.3 18.1	12.8 27.3	30.2 43.6	26.3 30.3

Values are given as the percent of initial radioactivity recovered as ${}^{14}CO_2$. The initial amount of $1 - {}^{14}C$ -glucose used was 22, 200 dpm. 150, ug folic acid added. A volume of 0.2 ml of a 1:1 diluted packed red blood cells was used per flask.

CONCLUSIONS:

From these results it is concluded that intact RBC's obtained from folate deficient QF rats are quite resistant to the folate deficiency even at a time when lejunal glycolytic enzymes had decreased activity. Repletion with folic acid led to little or no change in the RBC PPP activity in the intact QF cells, even though jejunal glycolytic enzyme activities increased.

RECOMMENDATIONS: None

PUBLICATIONS:

1. Rosenweig, N.S., R.H. Herman, F.B. Stifel and Y.F. Herman. The regulation of human jejunal glycolytic enzyme by oral folic acid. Jour. Clin, Invest. 1969 (in press).

2. Herman, Y.F., J.W. McHugh and R.H. Herman. The 1-¹⁴C-glucose metabolism of red blood cells of germfree rats on a folic acid-deficient diet. Proceedings of a Symposium: Gnotobiotic Research-Its Importance to basic mammallan research and Human medicine. Bth Annual Meeting of the Association for Gnotobiotics, Oak Ridge, Tennessee 1969.

STUDY NO. 6

Effect of a nickel-low, argininehigh diet on chicks,

PROBLEM:

Nickel has been shown to occur consistently in plant and animal tissues. Schroeder et al. (J. Chron. Dis., 15, 51 (1961)) have discussed the possibility that nickel may be an essential element playing a physiological role in the animal body. Among their reasons for this possibility were: 1) it shows biological activity in vitro, affecting certain enzymes, 2) it is present in plants and animals including the new born, 3) the existence of intestinal or hepatic barriers are implied, 4) it has little tendency to accumulate in tissues during a lifetime of exposure, and 5) some evidence for a role in pigmentation has been presented. Several investigators have attempted to establish nickel to be an essential element, employing methods of special diets, isotopic techniques, and nickel toxicity studies. None of these have resulted in definitive results indicating that nickel can play a biological role in vivo. Studies were initiated to determine whether or not nickel has a physiological role in the chick.

RESULTS AND DISCUSSION OF THE RESULTS:

Two experiments were conducted with day-old White Rock chicks without segregation according to sex. The birds were distributed at random into groups of 3 each in experiment 1, and 4 each in experiment 2, and placed in a plastic cage fabricated in a manner similar to that described by Schwartz and Smith (J. Nutr. 93, 182, (1967)). Three of these cages were placed into an all plastic isolator. These isolators were kept in a room in which the temperature range was 29° to 32°C.

The chicks in one isolator, 9 in experiment 1 and 12 in experiment 2, were fed a diet containing <0.08 ppm nickel on an air-dried basis (determined by atomic absorption spectrophotometry). A control group of chicks of the same size in each experiment was fed the same diet supplemented with 5 ppm nickel as NiCl₂. The diets used are described in Tables 1 and 2. The diet in experiment 2 had corn oil added to make it less hygroscopic. Additional arginine was added as it has been shown that high ievels of dietary arginine can affect a mineral deficiency. Also, additional vitamin D3 was added to the vitamin mix in experiment 2. Feed and glass-distilled water were provided ad libitum in polypropylene cups made from Erlenmeyer flasks.

When the chicks were 3 weeks of age, 5 chicks from each isolator were given 20 μ Ci of 63 Ni by gavage in the first experiment, and 9 chicks from each isolator were given 25 μ Ci of 63 Ni by gavage in the second experiment. Radioactive nickel as 63 NiCl₂, in 1 NHCl, was diluted with distilled water to give a stock solution of either 20 or 25 μ Ci 63 Ni/ml. Since the specific activity of the nickel was approximately 5 Ci/g, the doses were equivalent to approximately 4 and 5 μ g of nickel, respectively.

In experiment 1, 3 chicks from each group were killed 6 hours after isotope administration; 2 chicks, 24 hours after isotope administration. In experiment 2, 3 chicks from each group were killed at 6, 24 and 48 hours after isotope administration. All chicks were weighed, observed for abnormalities, and killed by decapitation. Blood was collected in a centrifuge tube containing 0,1 ml of heparin (100 units). A portion of the blood was separated into plasma and red blood cells fractions by centrifugation. Other tissues removed included tibia, kidney, spleen, liver, duodenum, gizzard, lung, muscle, skin, heart, aorta and feather. All tissues were frozen until ^{63}Ni analyses could be made. Length and width measurements of the tibias were determined by removing the flesh from the bone by rubbing with cheesecloth and measuring the smallest diameter and the largest length with calipers.

In both experiments, the level of nickel in the diet had no significant effect on the final average body weight of the chicks. The growth of the chicks was not considered fully normal in either experiment. This was attributed to the hygroscopic nature of the diet in experiment 1 and to the fact that some of the chicks showed signs of salmonella infection in experiment 2. Also, in both experiments, the chicks were not kept at an optinium environmental temperature due to the use of isolation equipment.

In both experiments, the level of nickel in the diet resulted in differences In gross appearances of the chicks. It appeared as if nickel had an effect on the pigmentation of the legs, and to a lesser extent, beaks of the chicks. The chicks which were fed the nickel-low diet had a bright gold color in these areas. In comparison, the chicks which were fed supplemental nickel had a darker gold color with a brown cast in their legs and beaks. Another difference which was noted was in the leg development of the chicks. The chicks fed the nickel-low diet had slightly thickened legs, and a larger joint area which appeared swollen when compared to those chicks fed supplemental nickel. The leg development in the nickel-low chicks was probably abnormal as these chicks appeared to walk with a stiffer gait and seemed to squat more often than those fed the supplemental nickel. In both criteria, leg development and pigmentation, the differences were more dramatic when the chicks were fed diet 2 which contained the higher arginine level.

Table 3 shows that the length:width ratios of the chicks fed the nickel low diet were significantly decreased when compared to those fed the supplemental nickel, thus giving further evidence that the leg bones in the nickellow chicks were thicker.

The results of the 63 Ni distribution study are presented in tables 4 and 5. The amount of, isotope retained per gram of tissue varied greatly among the tissues analyzed. Because of the variability and low number of degrees of freedom, few significant differences were found. In experiment 2, the nickel-low chicks retained a significantly greater amount of 60 Ni in the bone, liver, spleen, and aorta 6 hours after administration of the isotope. The isotope concentration was still significantly higher in the bone at the 48 hour time period. In contrast, the nickel-low chicks had significantly less isotope in the gizzard lining at the 6 and 24 hour time periods. There also appeared to be less retention of ⁶⁰Ni in the heart muscle at the 48 hour time period. In experiment 1, in which the chicks were not fed arginine, two significant differences were noted. At the 24 hour time period, the nickel-low chicks appeared to retain a greater amount of isotope in the primary sponglosa of the bone and in the feather. In both groups, several

tissues took up relatively large amounts of ⁶³Ni, including the bone, primary spongiosa, kidney, liver and aorta at the 6 hour time period. A large amount of isotope was still faund in the bone, kidney, and liver 48 hours after isotope administration. On the other hand, very little ⁶³Ni was found in the muscle, blood, red blood cells, and plasma after the 6 hour time period.

From the preceeding results, it appears that nickel may have a physiological role in chicks in respect ta pigmentation and leg structure. Kikkawa et al. (Science, 121, 43, 1955) proposed an hypothesis that color depended upon specific metals. They showed that in rabbit hairs, yellow contained nickel, titanium, iron and molybdenum and white contained nickel. From their in vitro indirect evidence, they concluded that nickel is important in white pigmentation. In vivo evidence that nickel plays a role in pigmentation in chicks has been described in the preceeding results. These results show that the level of nickel in the diet can affect the yellow colar af the chick legs and beak.

Othe: investigators have tried to find abnormalities in rats and mice fed nickel-low diets. Growth rate was one criterion most closely watched in these studies. With chicks, no significant difference in growth rate was noted. However, nickel appears to have an effect on bone formation. Nat only did the chicks appear to have thickened legs and swollen hocks, the length:width ratios of the tibias from nickel-low chicks were significantly less than those fed the nickel-high diet. The fact that leg abnormalities have not been noted in rats ar mice by previous workers is not surprising. Leg abnormalities normally occur in chicks fed zinc-deficient ar manganese deficient diets, whereas rats fed either of these types of diets usually show no gross leg defects. Perhaps a similar phenomenon occurs with nickel.

Due to the limitatians in the method of nickel determinatian, the level of nickel in the nickel-low diet is reported as < 0.08 ppm. From the data presented, it appears that the diet used was low enough in nickel to shaw that nickel prabably does have a physiological role in the chick. If the symptoms described are indications of a nickel deficiency in chicks, the deficiency is probably barderline. A high level of arginine in the diet appears ta enhance the borderline symptoms. Further attempts are being made to find a diet lower in nickel than the one presently in use.

It is also hoped that the exact level of nickel in the nickel-law diet can be determined. Then, the level of nickel needed to prevent any changes in pigmentation and leg structure can be ascertained.

CONCLUSIONS:

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Studies were conducted to determine whether or not nickel plays a physiological role in the chick. Chicks were maintained on low nickel diets (<0.08 ppm) and administered 63 Ni. Chicks fed nickel-low diets were observed to have (a) changes in the pigmentation of the legs, and to a lesser extent, the beak, and (b) to have legs with slightly swollen hocks and thicker bones. The length: width ratio of the tibias significantly decreased in the chicks fed the nickel-low diets. Retention of 63 Ni at the 6 hour time period after administration was greater in the bone, aorta, and liver of the chicks fed the low-nickel diet than in the nickel supplemented chicks. From the results, it appears that nickel has a physiological role in the chick.

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TABLE I

Diet Used in Experiment 1

Ingredient		G
Skim milk powder ^l		500.0
Corn meal, degerminated, enriched, yellow ²		475.1
Arginine		3.5
Glycine		10.0
Vitamin mix ³		I.0
Mineral mix ⁴		10.4
	TOTAL	1000.0

1. Nutritional Biochemicals Corp., Cleveland, Ohio.

2. The Quaker Oats Co., Chicago, Illinois.

3. The vitamin mix contained: (in mg) vitamin A palmitate (250,000 IU/g), 4.0; DL alpha tocopherol, powder (250 IU/g), 4.0; menadione, 0.5; pyridoxine · HCl, 1.0; folic acid, 0.9; and corn meal, 9896.0.

4. The mineral mix contained: (in g) $Ca_3(PO_4)_2$, 8.75; NaCl, 1.5; MnCO₃, 0.125; ZnO, 0.025; KI, 0.0005.

TABLE 2

Diet Used in Experiment 2

Ingredient		G
Skim milk powder ^l		500.0
Corn meal, degerminated, enriched, yellow ²		419.6
Arginine		30.0
Glycine		10.0
Vitamin mix ³		10.0
Mineral mix ⁴		10.4
Corn oil		20.0
	TOTAL	1000.0

I. and 2. - See Table I.

3. The vitamin mix contained: (in mg) vitamin A palmitate (250,000 IU/g), 4.0;
DL alpha tocopherol, powder, (250 IU/g), 4.0; menadione, 0.5; pyridoxine HCl, 1.0;
folic acid, 0.9; vitamin D₃ (40,000,000 IU/g), 0.01; and corn meal, 98,960.0.
4. The mineral mix as described in Table 1.

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Nutritional and Metabolic Aspects of Nutrients (Cant'd)

TABLE 3

Body Weights and Tibia Length-to-Width Ratios of Chicks Fed Different Levels of Nickel¹

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Experiment	Dietary Nickel	Body Weights ²	Length/Width Ratio af Tibia
	ppm	<u> </u>	
ł	0,08 ³	130 ^{a 4}	
I	5.00	128 ⁰	
2	0.08	121ª	17.62 ^a
2	5.00	109 ^a	18.38 ^b

1. Mean of 5 chicks in experiment 1, and 10 chicks in experiment 2.

2. Body weights were taken at 3 weeks.

3. Nickel content by analysis of diet with no supplemental nickel.

4. Values followed by the same letters within a given experiment are not significantly different (P > 0.10) from each other.

Distribution of 63 Ni in Selected Tissues at Time Intervals After a Single Oral Dose (Exp. 1)

		Hours A	fter Dose	
		<u>,</u>	24	2
······	Ni low ³	Ni high ⁴	NI low	Ni high
		% dose/g fro	esh tissue	
Bone	0.049	810,0	0.027	0.007
Epiphyseal plate Primary spongiosa	0,023 0,028	0,008 0,012	0,006 0,010 ⁵	0.002 0.003
Blood	0,024	0,066	0.018	0.01
Duodenum	0.073	0.170	0.026	0.024
Kidney	0,140	0,090	0.373	0,043
Spleen	800,0	0.021	0.016	0.016
Liver	0,025	0,013	0.033	0,006
Lung	0,02	0.028	0.084	0,022
Muscle	0.004	0,004	0.002	0,002
Skin	0,032	0.020	0.010	0.017
Heart + Aorta	0.031	0.014	0.010	0,003
Feather	0.041	0,052	0,028 ⁵	0.011
Gizzard lining	0,261	0.357	0.009	0.024

1. Mean, 3 animals/group.

2. Mean, 2 animais/group.

3. Nickel low indicates those chicks fed the basal diet containing <0.08 ppm nickel on an air dried basis.

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Nutritional and Metabolic Aspects of Nutrients (Cont'd) Table 4 (cont'd.)

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4. Nickel high indicates those chicks fed the bosal diet with a supplemental 5 ppm nickel.

5. Value is significantly different than the comparable value for the nickel high group within the same time period. (P < 0.10).

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Distribution of ⁶³Ni in Selected Tissues at Time Intervals After a Single Oral Dose

(Exp. 2)

			Hours Af	ter Dose		<u> </u>
		6	2	4	48	
	Ni low	Ni high ²	NI low	Ni high	Ni low	Ni high
			% dose/g	g fresh tissu	e ³	
Bone	0,2964	0.128	0,101	0.076	0.0984	0,041
Epiphyseal plate	0,102	0.070	0.023	0,024	0.015	0.017
Primary sponglosa	0,134	0.072	0,034	0,023	0.029	0.024
Hyaline cartilage	0.095	0,043	0.017	0,014	0.016	800.0
Blood	0,041	0,035	0.004	0,005	0.002	0.002
Red blood cells	0.021	0.015	0,002	0,002	0.002	0,001
Plasma	0,054	0,044	0,004	0,006	0,003	0.002
Duodenum	0.035	0.044	800.0	800,0	800,0	0,005
Kidney	0.609	0.292	0.291	0.073	0.193	10,141
5pleen	0.0444	0,023	0.021	0.020	0.017	0.010
Liver	0.1034	0.042	0.062	0.039	0.069	0.035
Lung	0.052	0.030	0.012	0.011	0.019	0,010
Muscle	0.018	0.017	0.003	0,004	0.003	0,003
5kin	0.051	0.048	0.019	0.022	0,023	0.023
Aorta	0.157	0,053	0.026	0.016	0,021	0,025
Heart Muscle	0.020	0.029	0.016	0,023	0.009	0,052
Feather	0,032	0,035	0.040	0.049	0,064	0.046
Gizzard lining	0,1024	0.198	⁴ 800.0	0.016	0.006	0,005

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Table 5 continued

ALIBERTATION IN A HALIDATE DE LA COMPANY A COMPANY A

1. Nickel low indicates those chicks fed the basal diet containing <0.08 ppm nickel on an air-aried basis.

2. Nickel high indicates those chicks fed the basal diet supplemented with 5 ppm nickel.

3. Mean, 3 animals/group.

4. Value is significantly different than the comparable value for the nickel high group within the same time period (P < 0.10).

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ABSTRACT

PROJECT NO. JA062110A822

Military Internal Medicine

WORK UNIT NO. 078

Analytical Biochemistry

Studies conducted under this work unit are as follows:

STUDY NO. 1 Analytical support and services

STUDY NO. 2 Development of analytical biochemistry procedures

Data handling system components have been implemented and modified, including further adaptation to specific analytical instrument functions, resulting in improved performance and subsequent better overall operational efficiency.

The scope and accuracy of services provided by the branch has been increased through the implementation, modification or development of several procedures. These included automated methods for serum a burnin, vitamin C, iron and total iron-binding capacity, and lipid phosphorus, and an electrophoretic method for abnormal hemaglobins. Modification of the components for an enzyme analytical system was initiated.

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BODY OF REPORT

WORK UNIT NO. 076

Analytical Blochemistry

STUDY NO. 1

Analytical Support and Services

PROBLEM:

The demands for support and service from this branch require an extremely high level of operating efficiency for both personnel and equipment. In order to achieve this, efforts must continually be made to modify existing analytical and data handling systems as well as to investigate new procedures. To expand services as required, additional automated analytical methods must be incorporated.

RESULTS AND DISCUSSION OF THE RESULTS:

1. The data acquisition system for the processing of analog information for gas chromatographs, amino acid analyzers and an enzyme analytical system has been implemented and improved. This included the final installation of a 32% disc storage unit and the installation and verification of operating programs to the gas chromatographs and unitso acid analyzers. The electronic integrating unit for the enzyme system was modified to meet the data handling requirements of this system.

2. The scope of services provided by the branch was broadened by the implementation of an automated analytical method for serum albumin and an electrophonetic method for abnormal hemoglobins in bload.

3. Several steps to improve branch performance have been initiated, cithough has yet entirely completed.

- a. Planning and ordering stages have been completed to: magnetic tape input for the analog computer, and for capacity for two additions channels from automated analysis systems to the on-line peak sorsing. logic system.
- b. Review and initial steps in the revision of the overall sample-input result-output procedure have been taken.
- c. Monual procedures for ash and Kijeldahi nitragen have been reviewed and initial steps taken to improve the accuracy and precision of these method.

Analytical Biochemistry (Cont'd)

CONCLUSIONS:

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Improvements and additions to the computer and data acquisition systems increase capacities for analytical procedures and data handling, while eliminating certain time-consuming steps. This permits an increase in services provided as well as time for performing and evaluating manual methods of analysis, overall procedure appraisal and method development.

RECOMMENDATIONS:

The data acquisition systems should be continually reviewed with the object of obtaining maximum performance to allow increasing capacity in an efficient manner. This should be augmented by further implementation and improvement of automated analytical methods, and improvement of the overall sample-results record keeping procedure to minimize back-log accumulation while broadening services. Personnel training should be included as an integral part of the program to maintain and improve efficiency of services.

STUDY NO. 2

Development of analytical biochemistry procedures

PROBLEM:

In order to furnish required support of increasingly sophisticated and complex research approaches, analytical methods and systems must be developed as specific needs arise. Adaptation of manual methods to automation must continually be investigated to achieve the degree of efficiency required for processing increased sample numbers.

RESULTS AND DISCUSSION OF THE RESULTS:

1. An automated method for vitamin C levels in blood has been developed, refined and implemented for analysis of survey samples. It involves a preliminary manual protein precipitation, and may be performed on serum or heparin plasma samples (volumes down to 100 microliters) in which it adequately covers the normal range of 0.2-2.0 mg/100 ml. It was discovered that the method will not work with EDTA plasma samples.

2. An automated procedure has been modified for determining serum iron content and total iron-binding capacity after manual saturation and absorptive removal

Analytical Biochemistry (Cont'd)

of excess iron. Iron is determined colorimetrically in 0.8 ml sample volume at the rate of 40 samples per hour using bathophenanthroline sulfonate as the chromagen (with slight changes from the method of Giovanniello et al.).

3. The system of analyses for evaluation of human lipid profiles was further refined with particular attention to lipid phosphorus determination. The automated analysis for low concentrations of inorganic phosphate of Hirsch et al. was adapted for use in this system to overcome a yield deficiency inherent to the previously used method.

4. Preliminary modification of standard components used in the enzyme analytical system have been initiated. Included is customization of the macro sampler with installation of a bath chamber and sample tube holders, alteration of the total cycle timer and sampling syphon, and connections to a digital integrator.

CONCLUSIONS:

Modifications and improvements in methodology increase the capacity and efficiency of the laboratory, and also the reliability of test results.

RECOMMENDATIONS:

Development of manual methods and particularly methods capable of automation and use with data acquisition systems should be continued since they are essential to the maintenance of laboratory efficiency and expansion of capacity, and subsequently enable the broadening of research programs.

PUBLICATIONS:

None.

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ABSTRACT

PROJECT NO.	3A062110A8222	Military Internal Medicine
WORK UNIT NO.	077	Nutritional Physiology

The following investigations have been conducted or initiated under this work unit during the past year:

STUDY NO. 1:	Renal and Liver Gluconeogenesis in Cold Exposed Rats (Further Experiments)
STUDY NO. 6:	Metabolic Effects of Starvation-Refeeding Stress in Swine
STUDY NO. 7:	Kidney Response to a High Protein Intake to Cold in the Rat
STUDY NO. 8:	Metabolic Effects of Glucagon and Adrenaline in Geese and Dogs
(Studies No. 2 -	• 5 were reported in previous Annual Reports)

Exposure of animals to a cold environment is associated with a severe loss of body weight. This loss reflects the negative caloric balance induced by the environment. These gross metabolic effects of cold exposure are accompanied by enhanced activities of enzymes involved in gluconeogenesis. Compared with the corresponding controls, cold exposure for two days causes an increase in the activities of glutamicpyruvic and glutamic-oxalacetic transaminases, glucose-6- and fructose-1, 6-phosphatases and phosphoenolpyruvate carboxykinase. These changes were observed in fed animals and in animals fasted for 48 hours. After eight days' cold exposure the activities of these enzymes had increased even further, but thereafter, their activities remained relatively constant.

Lipogenesis and gluconeogenesis were studied during both starvation and realimentation of adult male swine previously starved for periods up to 28 days. Enzyme activities associated with gluconeogenesis were markedly increased during the starvation period. In contrast, lipogenesis was minimal during starvation but increased during realimentation. These metabolic alterations are accompanied by systemic and pulmonary hypertension.

Experiments dealing with glucagon and adrenaline on the oxygen consumption and free fatty acid turnover in dogs and geese have been started.

BODY OF REPORT

WORK UNIT NO. 077 STUDY NO. 1 Nutritional Physiology

Renal and Liver Gluconeogenesis in Cold-Exposed Rats

PROBLEM:

When animals are first exposed to cold they must increase their rate of heat production, primarily by shivering, to compensate for an increased rate of heat loss. For several days they are not able to balance the caloric demands of the environment with an elevated caloric intake. In a sense, therefore, these animals are in a negative caloric state relative to their pre-exposure status: if they are growing animals, growth ceases; if they are adult animals, they lose weight. Thus, it would appear that during this acute stage of cold exposure metabolic activity is primarily directed toward maintaining the body thermal state at the expense of other less vital functions. The inadequate caloric intake and the consequent loss of tissue glycogen during acute exposure might promote alterations in intermediary metabolism which would favor an improved capacity for the oxidation of body lipid and protein. In addition, since carbohydrate is an important substrate in the metabolism of many tissues, we might also expect an improved capacity for gluconeogenesis. Accordingly, activities of several hepatic gluconeogenic enzymes, in vitro hepatic 14 CO₂ fixation, and in vitro renal gluconeogenesis were measured in rats exposed acutely or chronically to cold. In addition, concentrations of blood glucose plus liver and muscle glycogen in these animals were also determined.

RESULTS AND DISCUSSION:

Male, Holtzman rats, ranging in weight from 340 - 360 gm were used in all experiments. The control animals were maintained at $25\pm1^{\circ}$ C, the cold exposed animals at $5\pm1^{\circ}$ C for periods up to 30 days. A commercial rat diet and water were available ad libitum to all animals. Depending upon the experimental design, some animals were subjected to 48-hour fasting prior to experimentation, with only water available. At the end of the experimental periods, liver homogenates were assayed for the following enzymes: glutamicoxalacetic (GOT) and glutamic-pyruvic transaminases (GPT), glucose-6-phosphatase (GP), fructose-1, 6-diphosphatase (FP), phosphoenolpyruvate carboxykinase (PC).

Compared with the corresponding controls, cold exposure for two

Nutritional Physiology (Cont^{*}d)

days causes an increase in the activities of GOT, GPT, GP, FP, and PC. These changes were observed in fed animals and in animals fasted for 48 hours. After eight days' cold exposure the activities of these enzymes had increased even further, but thereafter, these activities remained relatively constant. A marked increase in CO₂ fixation was observed in rats exposed to cold for two days and this effect was maintained for periods up to one month. Cold exposure also enhanced the capacity of kidney cortex slices to form glucose from various gluconeogenic precursors. In general, gluconeogenesis was more pronounced in both fed and fasted rats at 5° C than in fed or fasted animals at 25° C.

RECOMMENDATIONS:

To further study basic mechanism underlying the foregoing metabolic alterations. These would include the effects of mobilization of free fatty acids, along with increases in adrenalcortical and thyroid activity on gluconeogenic capacity of cold-exposed animals.

STUDY NO. 6

Metabolic Effects of Starvation-Refeeding Stress in Swine

PROBLEM:

It is well known that the enzyme profile of the cell can be drastically changed by a great variety of dietary stimuli. For example, activities of several hepatic gluconeogenic enzymes increase during starvation and decrease to normal level, or below after refeeding with a normal diet. Conversely, activities of hepatic and adipose tissue enzymes involved in lipogenesis decrease during starvation and increase above normal upon refeeding. These, and other metabolic abnormalities accompanying refeeding after starvation may cause physiological stresses to become manifested either acutely or chronically. Since the domestic pig is biologically related to man in body and skeletal size, G. I. tract, choice of diet, etc., this species was chosen to study the effects of starvation and refeeding after starvation on a number of physiological and biochemical parameters.

RESULTS AND DISCUSSION:

Fifty male pigs, weighing 100 ± 15 lbs. were used. The animals, housed in individual pens, were divided into three treatment groups, as follows: 1. controls - fed ad libitum; 2. starved - for periods of

time up to 28 days with only water available; 3. starved-refed, starved for 28 days and refed for periods of time up to 16 days. The control and refed animals were fed a commercial pig diet, plus water, on an ad libitum basis. Prior to experimentation some animals were catheterized, and placed into metabolism cages to determine the effects of starvation-refeeding on the cardiovascular system. At the end of the experimental periods, the animals were sacrificed and the following parameters were studied: Activities of the key lipogenic and gluconeogenic enzymes in liver and adipose tissue and in liver and kidney, respectively, plus in vitro utilization of glucose-U- 14 C and acetate-1- 14 C by liver and adipose tissue. Compared with the controls, there was a gradual and marked increase in the incorporation of radioactivity into glycerol and fatty acid fractions and in the lipogenic enzyme activities throughout the whole refeeding period. At the end of the 16th day, activities of the following liver and adipose enzymes, respectively, were increased over the controls (in %); glucose-6phosphate dehydrogenase 78, 105; citrate cleavage 87, 135; NADPmalic dehydrogenase 98, 117; and acetyl-CoA carboxylase 110, 124. Incorporation of radioactivity into fatty acids increased over 200% during this period. | Lipogenesis was minimal at the end of the starvation period. In contrast, gluconeogenesis was maximal after 14 days of starvation. Heart rate was 60 beats/min during the control period, fell to 50 beats/min during the starvation period and rose on the 4th and 5th day of the refeeding period to 90 beats/min. During the same periods, arterial blood pressure changed from 120/80 to 110/70 to 145/120. Pulmonary hypertension developed during the refeeding period. Blood volume decreased during starvation period and returned toward the control value during the refeeding period.

SUMMARY AND CONCLUSIONS:

1. From Study No. 1 - Cold exposure increases the activities of hepatic key gluconeogenic enzymes both in fed and fasted rats. In addition, the capacity of kidney cortex to form glucose from various gluconeogenic precursors is also enhanced under this environmental stress. It would appear that accelerated mobilization of fatty acids from adipose tissue, along with increases in adrenocortical and thyroid activity promote the enhanced gluconeogenic capacity of the cold-exposed animal.

2. Study No. 6 - Gluconeogenesis gradually increases with the duration of starvation in swine, being maximal after 14 days of

starvation and, thereafter, remaining relatively constant. In contrast, lipogenesis was markedly increased in animals refed after starvation. These metabolic alterations are accompanied by increased heart rate and by pulmonary and peripheral hypertension.

RECOMMENDATIONS:

1. Further studies are required to delineate the cause(s) of hypertension that develops during refeeding.

2. Various diets should be investigated to determine the relative influence of dietary carbohydrate, fat and protein on the development and maintenance of hypertension.

STUDY NO. 7

Kidney Response to a High Protein Intake and to Cold in the Rat

PROBLEM:

Marked increases in kidney size are observed in animals exposed to cold or fed a high-protein diet. In fact, this effect of cold and diet appears to be additive, as evidenced by the markedly greater kidney enlargement seen in cold-exposed rats receiving a highprotein diet as compared to those receiving a high carbohydrate diet. Presumably, the cold-induced or diet-induced enlargement of the kidneys represents a compensatory response to an added functional load. In the case of high-protein diets, and probably in the case of cold-exposure where the caloric intake can be increased twofold, this added functional load may be caused by an increased metabolic turnover of nitrogen in the body. Accordingly, experiments have been designed to study various aspects of intermediary nitrogen metabolism as influenced by both cold exposure and high-protein diets. In addition, histological changes associated with kidney enlargement will be studied by light and electron microscopy.

RESULTS:

Experiments are in progress to study the effects of cold and/or high-protein diets in rats on various metabolic pathways which mediate or are related to protein catabolism.

RECOMMENDATIONS:

1. Complete present studies to examine the relationship of the responses of the kidney to high-protein diet and cold.

2. To perform renal function studies, such as creatinine clearance, urea clearance, glomerular filtration rate and renal plasma flow.

STUDY NO. 8

Metabolic Effects of Glucagon and Adrenaline in Geose and Dogs

PROBLEM:

The differences in the hormonal control of carbohydrate and lipid metabolism of the two classes of homoiotherms (i.e., birds and mannuals) have been recognized for many years. This is evident by the effects of glucagon (G) and adrenaline (A) on oxygen uptake of geese and dogs. Using unity (1) as the calorigenic effect in the hour after infusion of $3.0 \mu g/kg/min$ of λ into anesthetized dogs for 10 minutes, the other calorigenic effects of the same dose of A or G form the series: A in geose = 0; A in dogs = 1; G in dogs = 29; G in prese = 97. Experiments have been designed to analyze: a) the effects of a 10-minute infusion of glucagor, upon the oxygen uptake and plasma free fatty acid (FFA) turnover before and after pancreatectomy in dogs; (b) the effects of a 10-minute infusion of glucagon and adrenaline on blood sugar, blood lactate, plasma FFA, and plasma ketone bodies in geese; and (c) the effects of cold acclimatization on FFA turnover and the calorigenic action of glucagon in geese.

RESULTS:

Experiments are in progress studying the effects of glucagon or adrenaline infusions in geese and on the effects of glucagon on FFA turnover in dogs.

RECOMMENDATIONS:

1. Continue present studies to investigate the effects of glucagon and adrenaline in geese and dogs.

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effective in the female rats. **Parenteral** injection of sex steroids has no effect on the jejunal glycolytic enzymes. Folic acid to be effective must be given orally and has no effect when given parenterally.

We have studied one patient with symptoms of diarrhea, weakness, flushing, sweating, tremor and muscle spasm. This patient had a very mild megaloblastic bone marrow and elevated serum folic acid. It was proven that this patient has formiminotransferase deficiency of the jejunum to which we ascribe the gastrointestinal symptoms. The patient has improved on a high folic acid, low carbohydrate diet. We are currently investigating a number of other gastrointestinal problems which seem to be related to the inability of the individual's jejunal glycolytic enzymes to respond to folic acid and carbohydrate diets.

BODY OF REPORT

WORK UNIT NO. 078

Metabolic Response of Man to Nutrition or Disease

STUDY NO. 1

60 UK

Investigation of a Patient with Anterior and Posterior Hypopituitarism.

PROBLEM:

A patient with fatigue, weight loss, decreased libido, impotence, polyuria and polydypsia of five years duration was studied. It appeared that he had anterior and posterior hypopituitarism. He had sickle cell trait and the question was whether or not the hypopituitarism was related to the sickle cell trait.

RESULTS AND DISCUSSION OF THE RESULTS:

A 41-year old Negro male with fatigue, weight loss, decreased libido, impotence, polyuria and polydypsia of five years duration was studied. Symptoms began two months following a prolonged high altitude flight. His prostate and testes were abnormally soft. Visual fields and chromosome analyses were normal. A testicular biopsy showed no spermatogenesis with generalized tubular and interstitial tissue atrophy. Hemoglobin electrophoresis revealed A and S hemoglobins. A skull x-ray showed supracellular calcification but carotid arteriograms were normal. Craniotomy showed no evidence of tumor or other lesions. Response of his low urinary steroid excretion to ACTH and metopirone indicated secondary hypoadrenalism. Plasma growth hormore did not increase with insulin induced hypoglycen ia. Thyroid function was at the lower limits of normal. The values for serum and urine osmolality after overnight fasting, saline infusion, pitressin injection and prolonged water deprivation were diagnostic of pituitary diabetes insipidus. Review of the English medical literature revealed two patients with sickle cell trait and pituitary infarction proven at autopsy. Since the nephropathy of sickle cell trait is pitressin resistant, pitressin responsive hyposthenuria in persons with S hemoglobin suggests pituitary involvement. A causal relationship between this patient's sickle cell trait and generalized hypopituitarism is suggested,

CONCLUSIONS:

We conclude that the patient's antea was related to the sickle cell trait. .nd posterior hypopituitarism

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RECOMMENDATIONS:

Similar studies in patients with sickle cell trait and hyposthenuria should be done.

PUBLICATIONS:

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Pastore, R.A., Anderson, J.W. and R.H. Herman. Anterior and posterior hypopituitarism associated with sickle cell trait. Annals of Internal Medicine, 1969, in press.

STUDY NO. 2

The Effect of Diet on Gastrointestinal Enzymes.

PROBLEM:

The regulation of some gastrointestinal enzymes by diet has been discovered in the course of our studies. It is well known that dietary substances can increase enzymes in other tissues but the relationship of dietary substances to gastrointestinal enzymes has not been studied. We have engaged in a series of systematic studies in rat and man to elucidate the mechanisms whereby gastrointestinal enzyme levels can be controlled by diet and/or other substances.

RESULTS AND DISCUSSION OF THE RESULTS:

In normal man one can demonstrate that dietary sucrose and fructose can increase jejunal mucosal sucrase and maltase. There are several maltases in jejunal tissue one of which also has sucrase activity. It is this particular maltase that increases with sucrose feeding. Fructose when given in large amounts causes diarrhea in man, but in the few instances where it has been used it has increased jejunal sucrase and maltase. Jejunal lactase does not change with sucrose or fructose feeding. Because jejunal lactase has remained constant we have expressed our results not only in absolute values of sucrase and maltase but also in terms of the sucrase to lactase and maltase to lactase ratios. The time for increase in jejunal sucrase and maltase is from 2 to 5 days which is approximately that of the turnover of the jejunal epithelium cells of man. Thus, we postulate that sucrose and fructose affect the crypt cells of the jejunum. When the crypt cells migrate up the villus and form a brush border only then are the disaccharidase increases expressed. Large amounts of glucose also can increase sucrase and maltase but to a much lesser degree than isocaloric amounts of sucrose and fructose. Maltose and lactose have no effect on any of the disaccharidases.

> We next turned our attention to the effect of dietary sugars on glycolytic enzymes of jejunal epithelial cells. We determined that dietary glucose increases jejunal hexokinase, glucokinase, phosphofructokinase, glucose-1, 6-diphosphate aldolase and pyruvate kinase whereas fructose increases fructokinase, fructo-l-phosphate algolase, fructo-l, 6-diphosphate aldolase and pyruvate kinase. This was established both in rat and in man. In man, however, the fructose was in the form of sucrose. This result took only thirty minutes to become apparent, was quite evident at two hours and reached a maximum at about 12 hours. Thus, the effect seems to be directly on the jejunal epithelial cell and different from the effect on the crypt cell as seems to be the case with the disaccharidases. Dietary galactose increases the galactose metabolic pathway enzymes, galactokinase, galactose dehydrogenase, uridyl transferase and UDPG 4'-epimerase. Several of the jejunal glycolytic enzymes appear to decrease during galactose feeding.

Patients with tropical sprue were tested with dietary sugars and their glycolytic enzymes were found to respond very poorly. Since folic acid is a specific therapy for tropical sprue it was tested in such patients. Folic acid increased the glycolytic enzymes alone and even more so with diet. We then investigated the effect of folic acid in normal man and in rats. In normal man folic acid increases all glycolytic enzymes including fructokinase, fructose-l-phosphate and fructose-1, 6-diphosphate aldolases, phosphofructokinase, glucokinase, hexokinase and pyruvate kinase. In germ-free rats, kept on a folic acid deficient diet, gastrointestinal enzymes drop to low levels and gradually increase to normal as folic acid is given by stomach tube to the rats. This occurs at a time when the animals are not anemic and show no megaloblastic changes. Folic acid levels in the rats were quite low and gradually rose when folic acid was administered orally. The administration of folic acid parenterally has no effect. Only oral folic acid is effective on jejunal glycolytic enzymes.

We recently saw a patient who had symptoms of periodic diarrhea, weakness, tremor, flushing, muscle cramps, and pallor who had a mild megaloblastic bone marrow and elevated serum folic acid. The patient had formiminoglutamic aciduria with or without histidine loading. Her gastrointestinal enzymes did not respond to the usual doses of folic acid or to dietary carbohydrates. She had a marked carbohydrate intolerance which precipitated the symptoms. The patient was markedly underweight but had no evidence of gastrointestinal disease by histologic examination of jejunal tissue and no

evidence of malabsorption. Assay of formiminotransferase in red blood cells and in jejunum showed the enzyme to be deficient. Thus, this patient has a formiminotransferase deficiency of the jejunum. She has been treated with large doses of oral folic acid and a low carbohydrate diet and has improved markedly with increased well being and weight gain.

We are currently investigating other patients with obscure gastrointestinal problems and find that a number of them have poor adaptation of their jejunal glycolytic enzymes to diet and folic acid. Some of these individuals respond poorly to diet while others respond poorly to folic acid. The nature of these defects however, are not clear and are presently under study.

We have investigated the effect of various drugs and sex steroids on jejunal glycolytic enzymes. Rats given testosterone, stilbestrol. progesterone and estradiol orally show increases in phosphofructokinase and pyruvate kinase and a decrease in fructose diphosphatase. Fructose diphosphate aldolase, hexokinase and glucokinase were unchanged. In the male rat testosterone has the greatest effect with gradually decreasing effects occurring with estradiol, diethylstibesterol and progesterone. In the female rat estradiol was most effective with testosterone having a lesser effect. Therefore, not only is the type of sex steroid important but the sex of the animal is important in determining the response of the glycolytic enzymes. Parenteral sex steroids have no effect on gastrointestinal enzymes,

Other steroids have been studied in human volunteers. In obese patients undergoing starvation, we find that dexamethasone decreases the glycolytic enzymes and increases fructose diphosphatase and gluconeogenic enzymes which is in contrast to the sex steroids which increase the glycolytic enzymes and decrease the gluconeogenic enzymes.

Phenobarbital, on the other hand, increases all of the glycolytic enzymes. Neonycin, an antibiotic which can cause steatorrhea decreases all of the glycolytic enzymes.

CONCLUSIONS:

Dietary substrates have a profound effect on gastrointestinal enzymes in both man and rat. Varying sugars such as glucose, fructose, and galactose affect the specific enzymes that are important in their own metabolism. Substances such as folic acid and phenobarbital increase

all glycolytic enzymes and in the rat, sex steroids such as progesterone, testosterone, diethylstilbestrol and estradiol only increase some of the glycolytic enzymes and decrease fructose diphosphatase (a gluconeogenic enzyme) but has no effect on hexokinase, fructokinase, or fructose diphosphate aldolase. The sex of the animal also determines the degree of response to sex steroids. Folic acid increases all glycolytic enzymes as well and works orally but not parenterally. In the rats the sex steroids work only orally and not parenterally. We have elucidated the condition of formiminotransferase deficiency in the gastrointestinal tract and believe that this deficiency is responsible for the symptom complex seen in one patient. We are currently studying other patients with various obscure forms of gastrointestinal disease who also fail to respond properly to diet and folic acid. The nature of their diseases are less clearly delineated.

RECOMMENDATIONS:

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This is a most fruitful field for study which to date has elucidated a number of important findings with regard to the control of gastrointestinal enzymes. Our findings suggest that these regulatory mechanisms with regard to dietary substance are physiologically important and lead to gastrointestinal disease in man when defects in these mechanisms occur. This area of research must be continued and pursued diligently.

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ABSTRACT

PRÓJECT NO.	8A062170A822	Military Internal Medicine
WORK UNIT NO.	070	Radioisotope Support for
		Medical Research

During the past year the use of radioisotopes has continued to increase. An additional liquid scintillation counter and a gamma counting system have been purchased and are in use. An additional liquid scintillation counter and a gamma scanning system is now being used to help meet increasing counting requirements.

A master coding system has been developed in conjunction with the Computer Division to provide an information storage and retrieval system for biological data. A program has been written by the Radioisotope Branch to perform data reduction on information from the liquid scintillation counters for storage and use by the investigators.

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PROBLEM

The use at radioisotones has increased considerable during the last few wears and is still expanding. Thus, the amount of data and the requirement for valculations to be performed uppy these data continue to increase. The trend is travaté better and more complex counting systems. The Radioisotope Branch was established to develor and provide these systems and to maintair efficience in the utilization of costly counting instruments. This Pranch also precides procurement, storage and disposal of radioisotopes. Atomic Preces Commission licensing and Health Physics responsibility for the Latoratory.

RESULTS AND DESCEDSION OF RESULTS

The computer handling of radioisotobe data has been further interview. Nore efficient data reduction systems for the liquic scintillation counters are now in operation. New scintillation counting campusystems have been installed. These are now providing better serves to the haberatory. The transfer of the principal investigator has stopped further development of data handling systems.

SUMMARY AND RECOMMENDATIONS

The use hi the research constants for data reduction and for data storage and retrieval promises it significantly reduce the first spent in routine calculations. Additionally, the information is readily a valiable for more constructed relations and statistical respective. Continued work to more fully utilize the constants for data are seen would reem desirable.

A contral radioisothe countine facility browides the praximum utilizetion of equipment and pers as a central collecting facility for information on new instrumentation and counting techniques. Work should continue to improve the capabilities of this facility, wher a principal investigator is assigned to this Branch. Assignment of a full-time, trained investigator is imperative to perform adequate functioning of this work unit.

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ABSTRACT

PROJECT NO.	3A062110A822	N

WORK UNIT NO. 080

Virologic Clinical Research

The following investigations have been conducted under this work unit:

STUDY NO. 1: Rapid Diagnosis of Myxa- and Paramyxoviruses

- STUDY NO. 2: Nature of Hemagglutination by Parainfluenza, Virus Types 1, 2, 3
- STUDY NO. 3: Nature of the Immune Response Evoked by an Inactivated RSV Vaccine and After Natural RSV Infection
- STUDY NO. 4: Incidence of Hong Kang Influenza Among Personnel at the U. S. Army Medical Research and Nutrition Laboratory and Among Ambulatory Patients with Chronic Pulmonary and Cardio-vascular Diseases at Fitzsimons General Hospital
- STUDY NO. 5: A Field Trial of a Manovalent A₂ Hong Kong Variant Influenza Virus Vaccine
- STUDY NO. 6: A New Serologic Test for Respiratory Syncytial Virus (RSV) Using the Soluble Antigen Fluarescent Antibody (SAFA) Technique

 A satisfactory rapid diagnostic technique for the identification of myxoand paramyxovirus infections should have the following characteristics:

 capable af being completed within a few hours at as law a cast as possible;
 correlate highly with standard tissue culture isolatian and serolagic testing;
 not be dependent upon the presence of infectious virus. Two separate techniques are described which appear to meet these requirements: Direct (1)
 Hemagglutination (HA) and inhibition by typing sera; and (2) Fluorescent antibody technique utilizing respiratory secretion obtained at the time af illness. There appears to be a high correlatian (88-92%) with standard methods af identification.

2. A desirable characteristic of a rapid diagnostic technique is that demonstration of viral antigen be independent of the presence of infectious virus. Specifically, it is necessary to know if the direct microplate hemagglutination test utilizing respiratory secretion is capable of detecting noninfectious parainfluenza types 1, 2 and 3. Several experiments confirmed that HA by Para 1 is dependent and that of Para 2 not dependent upon the activity of infectious virus. In additional experiments it was demonstrated that HA by Para 3 also is dependent upon the activity of infectious virus. Noninfectious, Tween-80-ether (T-80-E) treated tissue culture fluid containing either Para 1, 2 or 3 antigens showed HA and could be serologically typed using the microplate method. Tween-80-ether treatment of respiratory secretion will be required to demonstrate noninfectious Para 1 and 3 by the direct HA technique described in Study No. 1. HA activity by noninfectious Para 2 can be demonstrated without treating secretion with T-80-E.

3. An altered host response to naturally acquired RSV infection requiring hospitalization was observed in 1966-67 and in 1967-68 among children 6-23 months of age at the time of initial immunization with an inactivated RSV vaccine in July 1966. Two RS vaccinees (ages 3 and 4 years) were hospitalized in 1969 with RSV pneumonia. The clinical course in each case seemed milder than that seen previously in atypical illnesses. The induction of delayed hypersensitivity by inactivated RSV vaccine was studied (1) in human recipients utilizing in vitro lymphocyte stimulation in response to vaccine antigens, and (2) in the guinea pig as an animal model. The mean per cent blastoid formation and mean uptake of radiouridine were greater in vaccinees than in controls in in vitro lymphocyte stimulation tests using RSV antigens. A delayed hypersensitivity reaction could be induced in guinea pigs by multiple injections of aqueous RSV vaccine mixed with complete Freund's adjuvant but not by aqueous vaccine alone.

4. A nationwide epidemic of Hong Kong Variant strain of A₂ influenza was experienced in 1968-69. Colorado was one of the states earliest and most severely involved. The first peak in reported cases in the Denver area occurred near the end of November. A much milder second peak was observed in mid-January 1969. No significant difference in attack rates for Hong Kong influenza was observed for adults living in the community who were (1) healthy and (2) afflicted with chronic respiratory and cardiac illnesses.

5. A field trial was conducted at the Colorado State Home and Training School in Denver in order to test the antigenicity and efficacy of a monovalent Hong Kong influenza virus vaccine in the presence of an epidemic. A smaller study was done at the Children's Asthma Research Institute and Hospital (CARIH) in Denver in order to determine the best routes of vaccine administration in order to afford maximum protection. About 80% of 98 vaccine recipients developed

4-fold or greater serum hemagglutination inhibition (HAI) antibady rises whether seronegative or seropositive prior to immunization. One parenterol injectian of vaccine was 45% protective in the presence of an epidemic. Parenteral followed by intranasal administration produced a geometric mean titer in the serum (G=212) higher than that produced by either 2 parenteral (G=77) or 2 intranasal (G=83) administrations. Hawever, 2 intranasal administrations produced a geometric mean titer per mgm IgA in nasal secretions (G=44.3) significantly higher than that produced by parenteral followed by intranasal administration (G=25.1).

6. It is desirable to develop a new serologic test for respiratory syncytial virus (RSV) infection suitable for studying the distribution of antibody in immunoglobulin fractions evoked by an RSV vaccine and after natural infection which avoids the cumbersome fractionation of materials by column chromatography. The soluble antigen fluorescent antibody (SAFA) technique is an indirect fluorescent antibody technique which would seem to fulfill this requirement. The test utilizes an RSV antigen produced in African green mankey kidney (AGMK) cells which is dried upon cellulose acetate discs, incubated with human serum at a single dilution of 1–20 and then allowed to react with fluorescein conjugated anti-human IgG, IgA or IgM globulins. Fluorescence is read objectively in a fluarometer. Twenty-five of 28 children with significant CF antibody responses were SAFA positive. Thirty-five of 39 CF negative pairs of sera were SAFA negative. The CF negative group included children with parainfluenza virus type 1, 2 and 3, adenovirus and mycoplasma pneumoniae infections diagnosed by standard isolation and serologic methads. The SAFA test represents a new, sensitive and specific serologic test for RSV infections.

BODY OF REPORT

WORK UNIT NO. 080

STUDY NO. 1

Virologic Clinical Research

Ropid Diagnosis of Myxo and Paramyxoviruses

PROBLEM:

Myxovirus and paramyxovirus infections are usually identified by standard tissue culture or egg isolations and demonstration of 4-fold or greater rises in several types of serum antibody. These procedures are costly, time-consuming and technically demonding. For natural reasons many positive viral identifications may be missed using standard techniques. Viral identification may be missed for technical reasons. In many epidemiologic surveys it is necessary to freeze specimens before inoculation. Myxovirus titers are known to drop even when specimens are stored at -70°C.

Rapid and alternative techniques for the identification of myxo- and paramyxovirus infections are needed. Chemotheropy for myxovirus infections is on the horizon adding further impetus to finding methods for rapid diagnosis.

RESULTS AND DISCUSSION OF RESULTS:

Two separate methods for rapid diagnosis of myxo- and paramyxovirus infections have been utilized to study directly nasal secretion obtained at the time of illness. Comparison with standard tissue culture isolations and serologic tests was made. Procedures for the initial processing of nasal secretion and techniques for direct hemagglutination and inhibition and fluorescent microscopy were described in the 1968 Annual Research Progress Report.

Application to Clinical Virologic Study (Tables 1-3)

Table 1 compares the demonstration of a hemagglutinating agent present in respiratory secretions by direct hemagglutination using the microplate method with identification using standard diagnostic methods. The rapid method detected 88% (15/17) of the identifications made by standard methods. Twenty-three secretions were HA negative from 25 children shown not to have such myxovirus infections by standard methods. Table 2 shows the distribution of specific agents identified by tissue culture isolation and/or a significant serologic response. The two infections missed by the rapid technique were caused by parainfluenza 3 and A₂ influenza. Typing was successfully done on 4 secretions containing Para-I or 2 as the single agent. Typing of A₂ influenza or combined infections has not yet been attempted.

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TABLE 1

RELATION BETWEEN DIRECT HEMAGGLUTINATION OF RESPIRATORY SECRETIONS AND STANDARD DIAGNOSTIC METHODS (42 SPECIMENS)

Standard N	Direct Hemagglutination				
		* .*	Positive	e Negative 2	
Virus Isolation	Positive	17	15		
Serologic Diagnosis	Negative	25	2	23	

TABLE 2

DISTRIBUTION OF SPECIFIC HEMAGGLUTINATING AGENTS DEMONSTRATED BY THE DIRECT HEMAGGLUTINATION OF RESPIRATORY SECRETIONS

	No. Identified By Standard Methods	No. Identified By HA	No. HA Negative
Paraflu, Type 1	1	1	-
Paraflu, Type 2	3	3	-
Paraflu, Type 3	1	-	1
A ₂ Influenza	6	5	1
Combined*	6	6	-
Total	17	15	2

* Dual Infections with 2 hemagglutinating agents or with one HA agent and a second agent not capable of hemagglutination with G. P. Erythrocytes

Table 3 shows the relation between direct immunofluorescent examination of exfoliated cells, serological response and isolation of respiratory syncytial virus. In patients with proven RS infections, 88.2% of nasopharyngeal aspirates and 87.5% of nasal smears, but only 72.7% of throat smears, were positive by immunofluorescent examination of exfoliated cells. Twenty-two (91.6%) preparations of exfoliated cells obtained by any of the 3 methods of collection from 24 patients with proven RS virus infections showed specific fluorescence. In patients where no virus was isolated and there were no significant antibody responses, 90.4% were negative by direct immunofluorescence. Specific fluorescent cells obtained from nasopharyngeol aspirates and in nasal smears correlated well with standard methods of identification with 94% overall agreement. Localization of RSV antigen in the cytoplasm showed the same characteristics in exfoliated cells as in tissue cells.

TABLE 3

Relation Between Direct Immunofluorescent Examination of Exfoliated Cells, Serological Response and isolation of Respiratory Syncytial Virus

Specimens and Standard Methods			Immunofluorescent Method		Overali Agreement
			Positive	Negative	
Throat Swab (41 Specime	ens)				
Virus Isolation and/or	Pos	22	16 (72.7%)*	6	
Antibody Response***	Neg	19	2	17 (89.4%)**	33∕41 (80 ,5°₀)
Nasopharyngeal Secretic (37 Specimens)	on				
Virus Isolation and/or	Pos	17	15 (88.2%)*	2	
Antibody Response	Neg	20	Ú	20 (100%)**	35/37 (94.6%)
Nasal Smear (17 Specim	ens)	·			2
Virus Isolation	Pos	8	7 (87.5%)*	١	
	Neg	9	0	9 (100%)**	. 16/17 (94.1%)
Exfoliated Cells by Any	Metho	od			2
ot Collection (45 Specin Virus Isolation and/or	Pos	24	22 (91.6%)*	2	
Antibody Response	Neg	21	2	19 (90.4%)**	41/45 (91.1%)

* % co-positivity; ** % co-negativity; *** 4X or greater CF rise

CONCLUSIONS:

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Two methods for rapid diagnosis of myxo- and paramyxo virus infections have been described. There appears to be a high correlation (88-92%) with standard methods of identification.

RECOMMENDATIONS:

These 2 methods show potential for routine rapid identification of myxovirus infections in diagnostic virology laboratories.

PUBLICATIONS:

- Nagahama, H., Eller, J. J., Stahl, M. K., and Fulginiti, V. A. Rapid diagnosis of myxovirus infections. 1. Diagnosis of respiratory syncytial virus infection by a direct immunofluorescent method. Abstract, American Pediatric Society, Atlantic City, N. J., April 1969, pp. 40.
- Eller, J. J., DeLeon, C., Stahl, M. K., and Fulginiti, V. A. Rapid diagnosis of myxovirus infections. II. Identification of Influenza A2 and parainfluenza types 1, 2 and 3 by the direct hemagglutination of respiratory secretion using a microplate method. Abstract, Society for Pediatric Research, Atlantic City, N. J., May 1969, pp. 108.

STUDY NO. 2

Nature of Hemagglutination by ParaInfluenza

PROBLEM:

If a respiratory secretion contains only noninfectious virus that will not hemagglutinate, treatment of the secretion with Tween-80-ether (T-80-E) offers a means of releasing HA antigen for detection. If, on the other hard, noninfectious virus will still hemagglutinate, treatment of secretion with T-80-E would not be necessary. A more detailed description of the problem was given in the 1968 Annual Research Progress Report.

RESULTS AND DISCUSSION OF RESULTS:

Several experiments confirmed that MA by Para 1, but not Para 2, is dependent upon the activity of infectious virus. Additional experiments demonstrated that MA by Para-S also is dependent upon the activity of infectious virus. Noninfectious, T-80-E treated tissue culture fluid containing either Para 1, 2 or 3 antigens showed MA and could be serologically typed using the microplate method described in Study 1.

CONCLUSIONS:

Tween-80-ether treatment of respiratory secretion will be required to demonstrate noninfectious Para 1 and Para 3 by the direct HA technique described in Study 1. HA activity by noninfectious Para 2 can be demonstrated without treating secretion with T-80-E.

RECOMMENDATIONS:

Identification of parainfluenza antigens in respiratory secretions treated with Tween -80-ether should be attempted.

PUBLICATIONS:

None.

STUDY NO. 3

Nature of the Immune Response Evoked by an Inactivated Respiratory Syncytial Virus Vaccine and after Natural RSV Infection

PROBLEM:

An altered host response to naturally acquired RSV infection requiring hospitalization was observed in 1966-67 and in 1967-68 among children & 23 months of age at the time of initial immunization with an inactivated RSV vaccine in July 1966. Those of the younger age group remaining in the area (now 2 1/2 to 4 year olds) were followed through another RSV season. Two RSV vaccinees (ages 3 and 4 years) were hospitalized in 1969 with RSV pheumonia. The clinical course in each case seemed milder than that seen previously in atypical illnesses. The induction of delayed hypersensitivity by inactivated RSV vaccine was studied (1) in human inecipients utilizing in vitro lymphocyte stimulation in response to vaccine antigens, and (2) in the guinea pig as an animal model.

RESULTS AND DISCUSSION OF RESULTS:

In vitro lymphocyte stimulation tests (Tables 4-5) (In collaboration with the Dept. of Biophysics, University of Colorado Medical Center, Denver). The mean per cent blast transformation and mean optake of radiouridine in cultures stimulated with RSV antigens were greater in RSV vaccinees than in age-matched controls. In both vaccinees and controls, per cent mitosis was almost negligible and most often zero. Tables 4 and 5 are expressed in terms of ratio of per cent blast transformation or radiouriditie uptake (CPM) with RSV antigen to no cantigen. Once again, the means and medians of these ratios were greater for RSV vaccinees than for age-matched controls. Nowever, the numbers of children studied were too small and standard deviations too large to show any statistical significance. Virolanie Clinical Remarch Nont'd

TABLE 4

RATIC OF PERCENT BLAST TRANSFORMATION IN CULTURES STIMULATED WITH TSV ANTIGENT TO THAT IN LINSTIMULATED CONTROLS

		Ratio So Blast	Transformation 13	V Antigen : No Antiger
Group	No.	Mean	Madian	Range
RSV Vaccinees	\$6	12.33	8. W	36. X - TO
Non-Voccinee	<i>[R</i>	1.50	7.72	Q.SD - 2 X

The determined at 72 - 742 hrs.; Thurditured to 1.92 dilution of retained sample of inactivated vervet matrices kidney grown RSV vaccine; The Cultures with he ant-Spen.

TABLE 5

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RATIO OF CAM* READKNENDINE UPTAKE) IN CULTURES STMULLATED WITH RSV ANTIGEN** FO CAM IN UNSTMULLATED CONTROLS***

		Rotio CPM RSV Antigen : No Antigen				
Croup	No.	Weban		Mediar	Pange	
RSV Vaccinees	22	` ₹, 39 -	.b.s s.ed.	7.21	D.36 - 2.50	
Non-Voccinees	71	1 ,29 a	¥9 s. 8.	7, 72	-0,32-3,80	

* Determined at \$6 - 144 hrs.; ** 3 (10 to 1 (1800 dilution of retained sample of inactivated vervet monkey kidney grown RSV vaccine; *** Cultures with no antigen.

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PUBLICATIONS:

None.

STUDY NO. 5

A Field Trial of a Monovalent A2 Hong Kong Variant Influenza Virus Vaccine (In collaboration with Dept. of Pediatrics, Univ. of Colarado Medical Center)

PROBLEM:

A field trial was conducted at the Colorado State Home and Training School in Denver in order to test the antigenicity and efficacy af a manavalent Hong Kong influenza virus vaccine in the presence af an epidemic. Gamma-A (IgA) antibady present in respiratary secretians is now felt to have the major rale in protectian against invasian by respiratary viruses. Methods of immunization producing the highest titers in secretions per mgm IgA are likely to be most successful. A smaller study was done at the Children's Asthma Research Institute and Hospital (CARIH) in Denver in order to determine the best routes of vaccine administratian in order to afford maximum pratection.

RESULTS AND DISCUSSION OF RESULTS:

Colorado State Home and Training School, Denver

Included in the study were 191 profoundly mentally and physically retarded children and a few adults wha were confined ta bed or had limited ambulation and were housed in three cottages and the Intensive Care Unit. Ninety-eight, or approximately half the residents were immunized and 93 served as unimmunized controls. The bed af a control usually was next to that of a vaccinee.

Lat No. 2MT16 (Eli Lily and Company) af a zonal centrifuged formalin inactivated, monovalent A₂/aichi/2/68 (Hong Kang Variant) influenza virus vaccine grawn in the extraembryanic fluid of embryonated hens' eggs was investigated. This vaccine was received in 5 ml vials and contained 800 CCA units per ml. 0.5 ml (400 CCA units) of vaccine was administered subcutaneously on November 19 and again on December 17, 1968. Venaus blood was obtained from each vaccinee and control prior ta each immunizatian and finally an January 28, 1969, six weeks after the second immunization. After the last bleeding, all recards of vaccinees and controls were reviewed far accurrence af fever or illness beginning December 1, 1968 (twelve days after the first injection of vaccine).

Antigenicity Data (Tables 6-7)

About 80% of vaccine recipients developed 4-fald or greater serum HAI rises whether seronegative or seropositive prior to immunization. By 6 weeks after the 2nd injection, 92% of vaccinees had seroconverted. Hawever, during

Vitelog & Clinical Research (Cont'd)

this period the attack rate for natural illness was high. The Geometric Meontiters (GMT) four weeks after the primary immunization were 32 for initially seronegative and 59 for initially seropositive vaccinees. The overall GMT for controls six weeks after the 2nd injection was 23 where there had been on attack rate af 47% for natural illness. Therefore, the comparable GMT for vaccinees of 77 reflects the antigenicity of the vaccine.

TABLE 6

SUMMARY OF ANTIGENICITY OF HONG KONG INFLUENZA VIRUS VACCINE BASED UPON \$4 X HAI ANTIBODY RISES IN PAIRED PRE- AND POST-IMMUNIZATION VENOUS BLOOD SPECIMENS

		>4-fold Rise in Post-vaccine Antibody					
Pre-Vaccine	• Antibody Status	4 Weeks after	1st Injection	6 Weeks after	2nd Injection		
		No.	%	No.	%		
Seronegative	(<8): vaccinees	45/57	(79)	54/57	(95)		
	controls	7/57	(12)	27/57	(47)		
Seropositive	(28): vaccinees	33/41	(80)	36/41	(88)		
	controls	2/36	(6)	17,/36	(47)		
Total	vacinees controls	78/98 9/93	(80) (10)	90/98 44/93	(92) (47)*		

*Attack rate 47% for notural illness by HAI tests.

TABLE 7

GEOMETRIC MEAN HAI TITERS*

				TITER	
				4 Weeks after	6 Weeks after
Pre-Vaccine Antibody Status		No.	Pre-Vaccine	1st Injection	2nd Injection
Sero negative	vaccinees	57	<8	32	59
	controls	57	<8	4	18
Seropositive	vaccinees	41	12	59	114
	controls	36	12	10	32
Total	vaccinees	98	4	42	77
	controls	93	4	5	23

*Titers rounded to nearest integer.

Clinical illness and deaths during 9-week influenza epidemic (Tables 8-9) Fever or definite febrile illnesses developed in 23% of 98 vaccinees as compared to 42% of 93 controls. Thus, the vaccine was 45% protective clinically. Since about 30% of the illness had already occurred by the time of the 2nd infection on December 17, the protective effect probably largely reflects that afforded by the 1st immunization. A protective effect from the booster could reasonably be expected by January 5. However, by that time 81% (Table 9) of the illness had already occurred. There were 3 deaths including 1 vaccinee and 2 controls

TABLE 8

	Vaccin	ees (98)	Controls (93)	
Illness Category	No.	%	No.	%
Definite fever	-			
OF	22	(22)	35	(38)
Definite febrile illness				
Questionable fever	1	(1)	4	(43)
Total	23	(23)*	39	(42)
Deaths	}	(1)	2	(2)

CLINICAL ILLNESS DURING 9 WEEK INFLUENZA EPIDEMIC AT COLORADO STATE HOME AND TRAINING SCHOOL, DENVER

*Vaccine 45% protective clinically.

TABLE 9

PERCENTAGE CUMULATIVE FREQUENCY OF ILLNESS* DURING 9 WEEK INFLUENZA EPIDEMIC AT COLORADO STATE HOME AND TRAINING SCHOOL, DENVER

Week		k	Comulative Frequency	Percentage Cumulative	
D	ate	<u>No</u> .	(No. of Patients)	Frequency	
Dec]**	1	6	10	
	8	2	15	24	
	15***	3	18	29	
	22	4	29 (1 death-∨)	47	
	29	5	42 (1 death-C)	68	
Jan	5	6	50	81	
	12	7	55 (1 death-C)	89	
	19	8	61	98	
	26	9	62	100	

*Defined as definite fever or febrile illness

and includes 5 patients with questionable fever.

**12 days after 1st injection of vaccine on November 19.

***Second injection on December 17.

Children's Asthma Research Institute and Hospital (CARIH), Denver Six older children institutionalized at CARIH each were given 0.5 ml subcutaneously of A₂ Hong Kong influenza virus vaccine on November 22, 1968. An intranasal booster was given on December 6 or 7. Six older children and 2 adults were given intranasal instillations on November 22, followed by intranasal boosters on December 6 or 7. Venous blood and nasal secretions were obtained from each vaccinee 11 to 14 days and again 6 1/2 to 7 1/2 weeks after the second immunization.

Serum and nasal HAI and nasal neutralizing titers after natural infection of A2 Hong Kong influenza (Table 10)

About 4 weeks after natural infection serum HAI titers were in the range of 128 to 1024 and seemed to be persisting at the same levels four weeks later. The HAI GMT in nasal secretions about 4 weeks after natural infection was 166 and dropped to 79.5 four weeks later. However, the HAI titer per mgm of IgA was 96.0 four weeks after natural infection ond had not changed four weeks later.

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TABLE	

SERUM AND NASAL HAI TITERS, NASAL NEUTRALIZATION TITERS AND I_{S}^{A} CONCENTRATIONS AFTER NATURAL INFECTION WITH A_2 HONG KONG "NFLUENZA

						NASAL			
SUBJECT	CEN	N.	4 We	eks' Po	st-infe	ction	8 Weeks'	Post-infec	tíon
	1#	۹ #2÷	lgA mgm/ml	HAI	Neut.	HAI IgA	lgA In∂m/ml	НАІ	HAI Agi
Ben.	512	512	3.25	512	>256	157.5	18.	128	158.0
Shab.	>1024	>1024	.38	256	200	673.7	.05	32	640.0
Sperl.	512	Z.†.	¢,	256	>256	324.0	N.T.	N.1.	
Stev.	>1024	>1024	3.68	512	512	139.1	1.61	128	79.5
Zetz.	128	N.T.	.57	Ø	9 1>	14.0	Z	х. Т.	
			Mean 1.37	GMT 166		96.0	Mean 0.82	GMT 79.5	97.0
*Appro	ximately 4 w	reeks after r	atural infect	ion.					

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**Approximately 8 weeks after natural infection.

Virologic Clinical Research (Cont'd)

Serum and nasal HAI titers and IgA concentrations after 2 prior administrations of A2 Hong Kong influenza vaccine. (Tables 11, 12)

Parenteral vaccine administration followed by an intranosal booster resulted in a quite high serum HAI GMT of 219 about 2 weeks after the booster. This method of vaccine administration resulted in a geometric mean titer in the serum approximating that produced by natural illness and was essentially unchanged about 5 weeks later. Intranasal administration of vaccine followed by an intranasal booster resulted in a much lower serum HAI GMT of 50 about 2 weeks after the booster which rose to 83 five weeks later. Both methods of vaccine administration resulted in geometric mean HAI titers per mgm of IgA in nasal secretions 2 weeks after the booster which were almost identical at 25.0 and 24.0. The HAI GMT per mgm IgA five weeks later produced by parenteral followed by intranasal administration was unchanged at 25.1. In contrast, the GMT per mgm IgA five weeks later produced by 2 intranasal administrations rose to 44.3. Parenteral followed by intranasal administration produces geometric mean titers in the serum higher than those by either 2 parenteral administrations or 2 intranasal administrations. However, 2 intranasal administrations produce a GMT per mgm IgA in secretions significantly higher 6 1/2to 7 1/2 weeks after the booster.

TABLE 11

Math	od of	11-14 2nd Im	Days after munization	6 1/2-7 1 2nd Im	/2 Weeks after munization
Vaccine Ad	ministration	No.*	HAI GMT	No.*	HAI GMT
Parenteral Intranasal	Nov. 22 Dec. 6-7	5	219	4	212
Intranasal Intranasal	Nov. 22 Dec. 6-7	5	50.1	5	83.1

SERUM GEOMETRIC MEAN HAI TITERS AFTER 2 PRIOR ADMINISTRATIONS OF A₂ HONG KONG INFLUENZA VACCINE

*Excludes those vaccinees who had natural influenza prior to initial immunization.

	11-14	Days after	2nd Immuniz	ation	6 1/2-7	1/2 Weeks of	ther 2nd Imm	un'zation
Method of Vaccine Administration	No.*	Mean IgA mgm/ml	Geometric Mean HAI	NA S	* .oV	Mean 19A mam/ml	Geometric Mean HAI	<u>¥</u>
Parenteral Nov. 22 Intranasal Dec. 6-	7 3	¢9.	16.0	25.0	*	ĸ	18.8	25.1
Intranasal Nav. 22 Intrana al Dec. 6-1	5	1.00	24.0	24.0	¢	2.01	89.1	14 .3
*Excludes those vacc	inees who	had natural	l înfluenza p	arior to in	nitial immur	nization.		

TABLE 12

Virologic Clinical Research (Cont'd)

CONCLUSIONS:

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One parenteral injection of vaccine was 45% protective in the presence of an epidemic. Parenteral followed by intranasal administration produced a GMT in the serum (G=212) higher than that produced by either 2 parenteral (G=77) or 2 intranasal (G=83) administrations. However, 2 intranasal administrations produced a GMT per mgm IgA in nasal secretions (G=44.3) significantly higher than that produced by parenteral followed by intranasal administration (G=25.1).

RECOMMENDATIONS:

It is desirable to study the rate of decay of antibody per mgm IgA in nasal secretions in serial samples obtained after natural illness and after immunization by the 2 different routes of vaccine administration.

PUBLICATIONS:

None.

STUDY NO. 6

A New Scrologic Test for Respiratory Syncytial Virus (RSV) Using the Soluble Antigen Elvorescent Antibody (SAFA) Technique

PROBLEM:

It is desirable to develop a new serologic test for RS virus infection suitable for studying the distribution of antibody in immunoglobulin fractions evoked by an RSV vaccine and after natural infection which avoids the cumbersome fractionation of materials by column chromatography. The soluble antigen fluorescent antibody technique in essence is an indirect fluorescent antibody technique which would seem to fulfill this requirement.

RESULTS AND DISCUSSION OF RESULTS: (Table 13)

The SAFA test utilizes an RSV antigen produced in African green monkey kidney (AGMK) cells which is dried upon cellulose acetate discs, incubated with human serum at a single dilution of 1 to 20 and then allowed to react with fluorescein conjugated anti-human IgG, IgA or IgM globulins. Fluorescence is read objectively in a fluorometer.

TABLE 13

FLUOROMETER READINGS IN SAFA TEST USING ANTI-Ig G COMPARED WITH CF RESULTS

Standard Tost	No. Paired	SAFA Test	Rises In Di	al Readings
Signuala resi	Jeiu	Medit	meululi	Kunge
CF Negative (<4-fold rises)	30	11	0	0 - 45
CF Positive (⊋4-fold rises)	28	3 0 6 165*	252 134*	57-745 57-351*

*Mean, median and range in rises excluding 10 prior recipients of an RSV vaccine.

The SAFA test was first standardized using anti-laG by testing 30 pairs of sera obtained from children hospitalized at Fitzsimons Generol Hospital for acute lower respiratory tract illness in 1967 which were RSV CF negative. The range of rises in fluorometer dial readings was 0 to 45, the mean was 11, and the median was 0. The CF negative group included children with parainfluenza virus types 1, 2 and 3 (17), adenovirus (2), A2 influenza (1) and mycoplasma pneumonia (1) infections diagnosed by standard isolation and serciogic methods. There was no evidence of cross reactivity with these common respiratory agents. An additional 9 pairs of CF-negative sera which were borderline were also tested using anti-IgG. One of these serum pairs showed a significant rise using the SAFA test, Three small infants from whom RS virus was isolated but who had CF-negative results were also negative by the SAFA test. In order to evaluate sensitivity 28 pairs of CF positive sera obtained from children hospitalized in 1967 and 1968 were tested using the SAFA technique. These patients included 10 RSV vaccine recipients hospitalized with RSV illness. The range in rises of fluorometer dial readings was 57 to 745, the mean was 306 and the median was 252. If the 10 RSV vaccinees are excluded the range was 57 to 351, the mean was 167 and the median was 134. Only 3 pairs of sera showed rises of less than 45 fluorometer dial readings. Twenty-five serum pairs were positive and 38 were negative by both methods giving an overall agreement of 94%.

CONCLUSIONS:

The SAFA test represents a sensitive and specific serologic test for RSV infections which can be utilized to study the distribution in the various immunoglobulin fractions of RSV antibody evoked by an RSV vaccine and after natural infection.

RECOMMENDATIONS:

The SAFA test should be used to study the distribution of serum antibody in immunoglobulin fractions. Further applications of the SAFA technique should be looked for.

PUBLICATIONS:

Eller, J. J., Moran, K. J., Gautsch, J. W., and Stahl, M. K. A new serologic test for respiratory syncytial virus infection using a soluble antigen fluorescent antibody technique. Presented of the Western Society for Pediatric Research, November 25–26, 1968, Denver, Colo. Abstract, Society for Pediatric Reseorch, Atlantic City, N. J., May 1969, pp. 100.

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ABSTRACT

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PROJECT NO.	3A062110A822	Military Internal Medicine
WORK UNIT NO.	081	A digital Computer Based Bio-Medical Information System to Support Special

A fully automatic, completely integrated biomedical information system is being developed to provide computer based medical support for Special Forces troops being deployed OCONUS and upon return to CONUS.

Forces Troops in the Field

BODY OF REPORT

WORK UNIT NO. 081

A Digital Computer Based Bio-Medical Information System to Support Special Forces Troops in the Field

PROBLEM:

The deployment of Special Forces troops generates a tremendous amount of medical information. This information consists of histories, physical examinations, laboratory findings and other data. The return of the troops provide an opportunity to evaluate the environmental factors encountered by the deployed troops. The manual manipulation of this amount of data is not feasible in the time frame required. Therefore, the need exists to develop the use of automatic data (ADP) equipment to assist the physicians in evaluating the data collected.

RESULTS AND DISCUSSION OF THE RESULTS:

The protocol for this application was developed as a cooperative study between the Surgeon's Office, Special Forces and the Computer Division, USAMRNL.

The current state of the project is:

1. The data transmission system (terminal and data phone) is installed and being debugged in reference to the specific project application.

2. The data preparation device is on-site and being used for input to the transmission system.

3. The data output device is connected and currently being debugged.

4. The data collection procedures have been revised and are being implemented on the computer.

5. Retrieval programs are developed.

6. Evaluation routines are currently being written.

A Digital Computer Based Bio-Medical Information System to Support Special Forces Troops in the Field (Cont'd)

CONCLUSIONS:

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The following conclusions have been drawn from the current state of the project:

1. The use of recall type data collection is not as reliable as on-site collection.

2. Data should be collected on an OCONUS basis as well as CONUS return.

3. Data transmission should be accomplished on a dedicated circuit.

4. At the current state of the art, no other method is as efficient for the defined tasks of storage, analysis, retrieval and display of the mass of collected data as the computer.

RECOMMENDATIONS:

1. The programming system should be extended to provide for complete interrogation procedures.

2. Data should be collected for the location in question, not from examination 30-60 days after the subject has returned to CONUS.

3. Upgraded communications facilities should be obtained.

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ABSTRACT

PROJECT NO.

3A062110A827

Military Environmental Medicine

WORK UNIT NO. 070

High Altitude Bioenergetics

A broad program investigating physiology and behavior of humans at high terrestrial altitude is in progress. We plan to locate and quantitate the performance decrements to be expected in soldiers in military operations at 10,000 to 14,110 feet; to measure the extent and the rate of acclimatization; to investigate the physiology, biochemistry, and pharmacology of the affected organ systems; and to ascertain how to minimize the decrements by selection, conditioning, previous environmental exposure, nutrition, drugs, or other variables. Acute mountain sickness and its prevention or treatment is a potentially serious military problem and will continue to be investigated. Recent investigations have shown that using liquid formula high carbohydrate diets resulted in a decrease in the clinical symptomatology during abrupt altitude exposure. Preliminary findings also appear to indicate that the energy requirements may be increased at high altitude since the oxygen uptakes during four levels of submaximal work on the treadmill were significantly increased at altitude.

BODY OF REPORT

WORK UNIT NO. 070

High Altitude Bioenergetics

PROBLEM:

The military necessity for physiological and behavioral studies at high terrestrial altitude became apparent with the Chinese invasion of India. Several members of this laboratory, because of their natural interests, the geographical location of the laboratory in Colorado, and the support of the Advanced Research Project Agency and the Medical Research and Development Command, began working on this problem in 1963. Our objective continues to be; to locate and quantitate the performance decrements to be expected in soldiers in military operations at 10,000 to 18,000 feet; to measure the extent and rate of acclimatization; to investigate the physiology, biochemistry, and pharmacology of the affected organ systems by selection, conditioning, previous environmental exposure, nutrition, drugs, or other variables. We plan to measure and correlate pulmonary, cardiovascular, metabolic, and biochemical parameters at rest, various levels of work and recovery in healthy human populations at both low and high terrestrial altitudes or with the use of altitude chambers or gas mixtures for short-term studies. We plan to participate in field studies with multiple performance measurements of actual military tasks and to study the effects of grade walking and load carrying.

RESULTS AND DISCUSSION OF THE RESULTS:

1. 1967 Study.

a. Two groups of men consumed a liquid diet of constant nutrient composition, with Group I consuming diet of normal distribution of nutrients and Group II, one with a high carbohydrate (68%) and low fat (20% of the calories). The data suggests that high carbohydrate diets and prior heavy physical activity at sea level may be beneficial in reducing the clinical symptomatology observed during rapid ascent to high altitudes. It also appeared that the high carbohydrate, low-fat diet greatly reduced the clinical symptoms at altitude over the control group. The V_{O2} 's in ml/kg/minute were all significantly increased at altitude for two groups during four submaximal work levels (3.5 mph on a 4 and 8% grade with and without a 20 kg pack) suggesting that energy requirements may be increased at high altitude.

b. At altitude, the significant body weight changes of 3,54 and 3,96 kg in Groups 1 and 11 were partitioned into significant losses of

High Altitude Energetics (Con't)

1.27 and 1.46 kg of fat, 1.77 and 1.85 kg of body water, 0.32 and 0.47 kg of body protein, and 0.16 and 0.18 kg of minerai, respectively. Body weight lost in excess of that attributable to the caloric deficit appeared to be due to a loss of body water. Blood and plasma volumes demonstrated significant decreases at altitude which also seems to indicate hypohydration of the body.

c. Nitrogen balances of 15 men receiving two different liquid diets were positive at sea level and negative at altitude but appeared to be partially a reflection of intake of both nitrogen and calories since dietary intake was greatly reduced at altitude. The first paper on mineral metabolism was published. In summary, sodium balances, exclusive of sweat losses, were positive at sea level and negative at altitude while potassium was retained at sea level and balances near equilibrium were observed at altitude. Calcium losses, attributed to low intakes, were observed throughout the study and magnesium balances were generally positive.

2. 1968 Study. During the summer of 1968, another study of human subjects was conducted to investigate further the possible beneficial effects of high carbohydrate intake on acute mountain slokness. This high altitude study differed from the earlier one in three aspects. First, eleven of the subjects were exercised vigorously throughout the study period. Second, the diet was only half liquid, rather than all liquid, and the remainder was solid in the form of TV dinners, etc. Third, carbohydrate metabolism was studied following an oral glucose load. Laboratory data are still being compiled from this study. Preliminary findings are as follows: for the first time, caloric intakes were decreased minimally at altitude, compared with substantial reductions noted in the earlier study. As a result, glucose tolerance curves, insulin secretion and serum free fatty acid concentrations during a standard oral glucose tolerance test were not altered significantly at altitude, The high carbohydrate, low fat diet was again noted to be beneficial in diminishing the symptoms of acute mountain sickness. The two non-exercising, normal diet-fed men became very ill at altitude and had to be removed from the study and taken to 1600 meters within 40 hours of exposure, while all of the eight high carbohydrate-fed subjects including two non-exercisers fared well. Some body weight, apparently water, was lost at altitude and then regained after the men returned to sea level. Nitrogen balances were positive in all except the non-exercising, normal-diet fed subjects. Samples of diets, urine and stools are presently being analyzed for sodium, potassium, calcium and magnesium, in order to determine the influence of high altitude and high carbohydrate intake on mineral balances.

fligh Altitude Energetics (Con't)

At altitude, the average weights of these exercised subjects feil moderately, but significantly in both groups. Changes in body water compartments were not significantly different between groups. Combined data (11 men) of total body water from DO dilution at altitude showed a significant decrement of 2,25 kg below sea level values, while extraceilular water (ECW) values remained unaltered. The calculated intraceilular (ICW) compartment was significantly diminished at altitude by 3,25 kg below sea level value. These data show that, at altitude, men who exercise strenuously and maintain high caloric intake develop a significant diminution in total body water secondary to reduction of the ICW compartment.

CONCLUSIONS:

1. In the 1967 study, using a liquid high carbohydrate diet, the following was observed:

a. Heavy exercise prior to altitude exposure was greatly beneficial in reducing the clinical symptomatology.

b. Negative nitrogen balances resulted from acute exposure to altitude.

c. The decreased dietary intake at altitude resulted in a significant body weight loss which was not all due to the caloric deficit but to the body water loss. Other factors such as a significant decrease in plasma and blood volume and negative water balances indicate hypohydration.

2. In the 1968 study, the men were highly motivated and consumed practically all of the diet at altitude. This resulted in positive nitrogen balances, normal glucose tolerance curves, insulin secretion and free fatty acids at altitude. Additional information on the hypohydration effect at altitude was obtained. The body weight losses were significantly decreased. A decrease in total body water, without change in ECW, was measured which reflected changes in intracellular water.

RECOMMENDATIONS:

1. Future studies will include changes in plasma lipids, lipoproteins, glucose, lactate and pyruvate, glucose tolerance and metabolic patterns during a standard submaximal exercise test.
High Altitude Bioenergetics (Con't)

2. Specific metabolic studies in human subjects receiving different diets will attempt to evaluate at high aititude alterations in carbohy-drate and lipid metabolism. The studies will include:

a. Intermixing glucose mass, apparent distribution space of glucose, and rates of appearance (hepatic output) and disappearance (tissue uptake) of glucose, measured by the technique of a measured tracer injection of glucose -UC⁴.

b. Influence of physiologic dose of glucagon on concentrations of plasma glucose, insulin, growth hormone, and free fatty acids.

c. Influence of intravenous infusion of the amino acid arginine on plasma glucose, insulin, growth hormone, and free fatty acids.

d. Metabolic pattern during submaximal exercise.

e. Continuation of mineral metabolism studies of humans during altitude exposure, with various other environmental stresses and with altered amounts of macro nutrients as indicated.

3. Studies at altitudes above 14, 100 feet in altitude chambers, or in South America.

4. Studies of altitude and cold adaptation.

High Aititude Bioenergetics (Con't)

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- Consolazio, C.F., L.O. Matoush, H.L. Johnson, H.J. Krzywicki, and G.J. Isaac. The effects of high carbohydrate diets during abrupt altitude exposure (4300 Meters). Performance and Subjective Symptomatology (Clinical). Proc. of the International Symposium on Altitude and Cold. Aspen, Colorado. Fed. Proc. 28: 937-943, May-June, 1969.
- Johnson, H. L., C. F. Consolazio, L. O. Matoush, and H. J. Krzywicki. Nitrogen and Mineral Metabolism at Altitude. Proc. of the International Symposium on Altitude and Cold, Aspen, Colorado. Fed. Proc. 28: 1195-1198, May-June, 1969.
- 4. Carbohydrate Metabolism in Men at High Altitude. Alfonso Janoski, H. L. Johnson, and S. S. Sanbar. Fed. Proc. 28: 1858, May-June, 1969. (Abstract)
- 5. The Effects of Diet and Exercise Upon Men Exposed to Altitude. H.L. Johnson, C.F. Consolazio, H.J. Krzywicki, and T.A. Daws. Fed. Proc. 28: 1859, May-June 1969. (Abstract)
- 6. Changes in Body Water Compartments at High Altitude.
 H.J. Krzywicki, C.F. Consolazio, H.L. Johnson and
 W.C. Nielsen. Fed. Proc. 28: 2218, May-June, 1969.
 (Abstract)

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PROJECT NO.	3A062110A827	Military Environmental Medicine
WORK UNIT NO.	07 1	Cardiovascular and Pulmonary Responses at High Altitude

The following investigation has been done under this work unit:

STUDY NO. 1: Hemodynamics and Blood Variables at Altitude

Nine human volunteers were studied initially at sea level and then for 14 days (14, 100 ft.) altitude. Hemoconcentration attributable to a reduction in plasma volume occurred during the first five days at altitude. Blood gas and pH declined exponentially during the 14 days at altitude. Heart rate and cardiac output were increased at two days' exposure, then declined toward control levels at 14 days. Stroke volume was unchanged at two and five days. At 14 days, it was below control values. Mean arterial blood pressure rose slightly on the second day at altitude and remained elevated for the 14-day period. These data indicate that the discrepancy in the literature is accounted for by differences in the length of time at altitude.

BODY OF REPORT

WORK UNIT NO. 071

STUDY NO. 1

Cardiovascular and Pulmonary Responses at High Altitude

Hemodynamics and Blood Variables at Altitude

PROBLEM:

Previous studies from this and other laboratories have produced apparently conflicting results with respect to the changes in hemodynamic function associated with chronic high altitude exposure. Included in these conflicts are data showing the resting cardiac output and stroke volume to be increased, unchanged and reduced as a consequence of altitude exposure. It seemed possible that much of the conflicting data could be attributed to differences in the elevations at which various studies were conducted or to differences in the duration of exposure. As part of another field study (Study No. 1, Work Unit 072), the second of these possibilities was investigated.

RESULTS:

Nine human volunteers were studied initially at low altitude in San Antonio and subsequently after altitude exposure on Pikes Peak for 2, 5 and 14 days, Hematocrit, hemoglobin and plasma volume measurements revealed an abrupt hemoconcentration during the first five days of altitude exposure. This was attributable to a reduction in plasma volume. Between five and 14 days' exposure hemoglobin and hematocrit values increased more gradually, presumably because of an altitude-induced increase in hematopoietic activity. Blood gas and pH measurements revealed an exponential decline in arterial P_{CO2} and bicarbonate concentration, i.e., rapid decreases during the early stages of exposure and more gradual decrease thereafter. Minimal arterial PO2 values were observed on the second day of exposure with no evidence of recovery thereafter. Because of the increase in ventilation and hemoglobin concentration arterial O₂ saturation and O₂ content, particularly the latter, exhibited marked recoveries from the low values exhibited on the second day at altitude. Normal values for arterial O₂ content were thus observed after 14 days on Pikes Peak.

Hemodynamic measurements revealed a marked increase in resting, supine heart rate and cardiac output on the second day of altitude exposure. Stroke volume was unchanged at this time. With longer exposure heart rate decreased, but did not reach control values, Cardiovascular and Pulmonary Responses at High Altitude (Cont'd)

even after 14 days on the Peak. Stroke volume reverted to normal values after two and five days[†] exposure and was subnormal after 14 days. Mean arterial pressure measurements showed a slight increase on the second day of altitude exposure and maintenance of this increase over the course of the altitude sojourn.

CONCLUSIONS:

The major discrepancies in the literature concerning acid-base and hemodynamic functions at altitude can be ascribed to differences in duration and level of altitude exposure, particularly the former. The data obtained in this study suggest temporal relationships between arterial P_{CO2} , bicarbonate concentration and O2 content and the severity of some altitude sickness symptoms.

RECOMMENDATIONS:

1. Studies should be conducted to establish or refute mathematically the foregoing suggestions about mountain sickness and blood composition.

2. However, due to loss of two civilian scientists because of the manpower reductions imposed on the Laboratory, to a lesser extent, the reduction in financial support and, to effect better management of resources, research under this work unit will be discontinued. Future efforts in cardiopulmonary physiology at high altitude will be incorporated into Work Unit 073.

PUBLICA TIONS:

1. Shields, J.L., J.P. Hannon, C.W. Harris & W.S. Platner. Effect of Altitude Acclimatization on Pulmonary Function in Women. J. Appl. Physiol. 25:606-609, 1968.

2. Hannon, J. P., J. L. Shields, C. W. Harris. Effects of Altitude Acclimatization on the Blood Composition of Women. J. Appl. Physiol. 26:540-547, 1969.

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ABSTRACT

PROJECT NO. 3A062110A827 Military WORK UNIT NO. 072 Metaboli

Military Environmental Medicine Metabolic Effects of Altitude

The following investigation has been conducted under this work unit:

STUDY NO. 1: Fluid and Electrolyte Shifts at Altitude

Experimental work was directed to the effects of fluid shifts during acute altitude exposure on electrolyte metabolism. Serum Na, K, Ca, Mg, Cl, PO_4 , HCO_3 , and protein anion were measured in nine volunteers initially at sea level and after 1, 3, 7 and 14 days at 14, 100 ft. altitude. Calculations of total extracellular ionic content revealed marked loss of Na and HCO_3 and lesser losses of Cl, K, and Ca. These data indicate a major shift of extracellular electrolytes from the extracellular to the intracellular compartment during the initial stages of high altitude exposure.

BODY OF REPORT

WORK UNIT NO. 072

Metabolic Effects of Altitude Fluid and Electrolyte Shifts at Altitude

PROBLEM:

STUDY NO. 1

Previous work on both animals and humans revealed marked shifts of body fluid from the vascular and interstitial compartments to the intracellular compartments. The mechanisms responsible for these shifts are unknown. Also unknown are the effects of these fluid shifts on electrolyte metabolism. Experimental work was therefore directed at the second of these unknowns.

RESULTS AND DISCUSSION:

The serum concentrations of Na, K, Ca, Mg, Cl, PO₄, HCO₃, and protein anion were measured in nine soldier volunteers, initially at low altitude and subsequently after 1, 3, 7 and 14 days at Pikes Peak. In addition, total body water and the extracellular space were measured as indicated in the 1967 Annual Research Progress Report. Intracellular space was determined by difference. These measurements revealed the serum concentrations of Na and Ca to be unaffected by altitude exposure while the concentrations of K, Mg, Cl, PO₄, and protein anion were increased and the concentration of HCO₃ was decreased. The reduction in serum bicarbonate concentration was offset by the increases in Cl, PO₄, and protein anion, the major compensation being attributable to the elevation in Cl concentration. Calculations of the total amount of extracellular ions revealed marked loss of Na and HCO₃, and lesser losses of Cl, K and Ca.

In a second series of experiments conducted on ten soldier volunteers maintained on mineral balance and constant intakes of Na and K, high altitude exposure was associated with little or no change in Na excretion, a positive K balance, an increase in the urinary Na/K ratio, an increase in cortisol excretion, a reduction in aldosterone secretion, but little or no change in plasma renin concentration.

CONCLUSIONS:

The foregoing data suggest a major shift (up to 25% of the total) of extracellular compartment. The causes of this shift, in fact the specific site or sites of this shift, are unknown.

Metabolic Effects of Altitude (Cont⁴d)

RECOMMENDATIONS:

1. Conduct long-term animal studies to determine the time required to reestablish the normal fluid compartment sizes and compositions seen in high altitude native populations. This will probably require exposure of one to three months.

2. Also needed are animal studies directed toward delineating the specific tissue sites where the fluid and electrolyte shifts occur. Finally, the fundamental mechanisms responsible for these abnormalities need to be explored.

3. However, due to the loss of two civilian scientists because of the manpower reductions imposed on the laboratory, to a lesser extent the reductions in financial support and to effect better management of resources, research under this work unit will be discontinued. Future efforts will be incorporated into Work Unit 073, Physiological, Metabolic and Psychological Aspects of High Altitude Exposure.

PUBLICATIONS:

1. Hannon, J. P., J. L. Shields, C. W. Harris. Effects of Altitude Acclimatization on the Blood Composition of Women. Am. J. Physiol. 26:540-547, 1969.

2. Hannon, J. P. and J. L. Shields. Effects of Acute Altitude Exposure on Serum and Extracellular Electrolytes. Fed. Proc. 28:594, 1969. (Abstract)

3. Janoski, A. H., B. K. Whitten, J. L. Shields and J. P. Hannon. Electrolyte Patterns and Regulation in Man During Acute Exposure to High Altitude. Proc. of the Inter. Symp. on Cold and Altitude. Fed. Proc. 28:1185-1189, 1969.

4. Hannon, J. P., J. L. Shields and K. S. K. Chinn. Effects of Acute High Altitude Exposure on Body Fluids. Proc. of the Inter. Symp. on Cold and Altitude. Fed. Proc. 28:1178=1184, 1969.

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ABSTRACT

PROJECT NO. 3A0621

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WORK UNIT NO. 073

Military Environmental Medicine

Physiological and Psychological Aspects of Performance at Altitude

The following investigation has been initiated under this work unit:

STUDY NO. 61 An Analysis of the Physiological, Biochemical and Psychological Factors Underlying Acute Mountain Sickness

This work unit is designed to evaluate various impairments that occur during temporary residence at high altitude. The symptoms of Acute Mountain Sickness (AMS) are usually manifest after several hours' exposure to altitude and reach their maximum intensity within 24 to 48 hours. Thereafter, symptomatology decreases with minimal levels being achieved after 4 to 7 days. AMS can be a severe and incapacitating experience to unacclimatized individuals rapidly exposed to high altitude. Reevaluation of data from previous studies has shown that the General High Altitude Quustionnaire (GHAQ) has proven its validity as a reliable measure of the estimate of AMS. Some physiological and blochemical variables have been shown to be correlated with individual symtpoms indicated in the GHAQ. This data analysis was used as the background to develop a study of normal human volunteers at sea level and high altitude (14, 100 ft.) to examine the relationship between the symptomatology of AMS and the altered physiological and biochemical phenomena attendant to high altitude residence.

BODY OF REPORT

WORK UNIT 073

Physiological and Psychological Aspects of Performance at Altitude

STUDY NO. 6

An Analysis of the Physiological, Biochemical and Psychological Factors Underlying Acute Mountain Sickness

PROBLEM:

Much descriptive information has been gathered regarding alteration of various blochemical and physiological phenomena during acute exposure of humans to high terrestrial environments. However, little is known about the relationship of these changes to the symptomatology of AMS. The objective of this study is to determine which alterations, acting either alone or in combination, are correlated with the observed changes in the symptomatology of AMS.

RESULTS:

This study is planned for next summer. Normal human volunteers will be studied at sea level and for five days at altitude. A large number of measurements potentially relevant to AMS will be studied. Through the use of multivariate statistical procedures, those variables most relevant to the etiology of AMS should be determined.

RECOMMENDATIONS:

1. Information to provide a better physiological and blochemical rationale to design specific experiments that will elucidate the mechanism(s) of AMS is needed.

2. Due to the loss of two civilian scientists because of manpower reduction imposed upon the laboratory, and to a lesser extent the reductions in financial support, research under this work unit will be curtailed. Future basic studies in cardiopulmonary physiology, metabolism and performance at altitude will be consolidated in this Work Unit 073 which will be retitled, Physiological, Metabolic and Psychological Aspects of High Altitude Exposure.

PUBLICATIONS:

1. Carson, R. P., W. O. Evans and J. L. Shields. Discussion of Some Clinical Aspects of Acute Mountain Sickness. Biomedical Problems of High Terrestrial Elevations. A. H. Hegnaner (Ed.) Physiological and Psychological Aspects of Performance at Altitude (Cont¹d)

U.S. ARIEM, Natick, Mass., pp. 9-23, 1969.

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2. Hannon, J. P., J. L. Shields and C. W. Harris. Anthropometric Effects of High Altitude Acclimatization in Females. Am. J. Physical Anthropology (In Press).

3. Shields, J. L., J. P. Hannon and R. P. Carson. Pathophysiology of Acute Mountain Sickness. Biomedical Problems of High Terrestrial Elevations. A. H. Hegnauer (Ed). U. S. ARIEM, Natick, Mass. pp. 24-31, 1969.

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ABSTRACT

PROJECT NO. 3A062110A830

WORK UNIT NO. 061

Bio-Sensor Systems

Nutritional Aspects of Military Dog Performance

STUDY NO. 1

Ft. Benning Field Study

This study was to determine whether Standard Item dog foods (dry), purchased under Federal Specification, would provide sufficient calories to permit working military dogs to maintain weight and work efficiency. Many commanders and veterinarions caring for such dogs had reported loss of weight and insufficient stomina in them, especially in warm, humid climates, e.g., S.E. Asia and Ft. Benning, Ga.

Sixty dogs from a dog class at the Scout Dog Training School, Ft. Benning, Ga., were subdivided into groups of 20. One group was fed a specially formulated high colorie, highly digestible dry ration, Military Stress Diet 198 (MSD); the other two groups each received one of the two Standard Item dry dog food products, one supplied by the Sturdy Dog Food Co., (Sturdy), one by Quaker Oats Co., (Gaines). The dogs were trained in the usual manner. The study was performed during July, August and September (12 week training cycle) to maximize effects of heat.

Information was sought in three generol areas: food intoke and weight performance, relative digestibility and nutrient value of the rations, and some effects of heat.

A constant, measured amount of food was offered each day, and the amount consumed was recorded. Dags were weighed weekly and blood was obtained weekly for determination of levels of electrolytes, enzymes, other metabolites, proteins and complete blood counts. Ambient temperature and humidity were recorded hourly and body temperature of the dogs was determined under various work conditions and at feeding time. Daily water consumption was estimated, as was relative hydration of the dogs before and after work. At the end of the 12-week study, four dogs from each group were placed in metabolic cages and relative digestibility of each ration was determined by proximate analysis and bomb calorimetry of food and feces.

MSD was clearly superior to the Standard Item rotions. The dogs ate less of it and gained weight while those on the other diets lost weight. MSD contains approximately 50% more calories as digestible energy and each of the components measured by proximate analysis (protein, fat, carbohydrates, ash, dry matter) was more digestible by 10-20%. Not only did MSD contain more calories, but digestibility was 94% as compared to 80% for the other two diets.

Heat exhaustion among the dogs was evident, especially early in the training period. During road marches, approximately 10% of the animals were unable to maintain thermal equilibrium when the ambient temperatures were in the 80's and relative humidity 50-75%. The working temperature under these conditions for most dogs was 103–105°F; those dogs not equilibrating continued to increase body temperature above 106°F; these became weak and ataxic, could not keep up and had to be rested and "wet down" to reduce their temperature. Occasional animals would collapse with temperature over 107°F. One died, after achieving a temperature above 108°F. The factors which determine whether a given dog would be able to equilibrate (primarily dissipate heat at an adequate rate) on a given day are not clear. Rate of heat gain (speed of march, ambient temperature and humidity) is important, but the animals which fail to dissipate sufficient heat, primarily by evaporative cooling of oropharynegeal mucosa, are different in some yet-undescribed way. Obvious impairment of panting (jerking on collar or choke chain; muzzle), excitement (barking and lunging) and water deprivation are certainly factors. Effects of kidney function and acid-base control may also be important. Along with many other factors, hyperthermia decreases the dogs' responsiveness to handlers and undoubtedly decreases training efficiency as well as scouting performance.

A ration having the digestibility and caloric density of MSD is recommended for standard procurement for military use, either for special situations or for routine use. Two pounds/day of the Gaines product would be required to provide caloric balance, but the dogs in this study averaged only 1.7 lbs/day. The Sturdy product is not recommended for military use due to low acceptability by the dogs, and low digestibility.

At least 50 Kcal/lb of body weight/day must be absorbed under the conditions of this test for these dogs to maintain body weight.

BODY OF REPORT

WORK UNIT NO. 061

Nutritional Aspects of Military Dog Performance

STUDY NO.

Et. Benning Field Study

PROBLEM:

Despite careful selection and training of today's military dog, operational reports and individual observations indicate that substantial numbers of dogs do not perform at the levels anticipated. Lack of endurance and failure to maintain physical condition seem to be the most frequent complaints. Many veterinary officers have suspected the cause to be nutritional and believe the rations offered these dogs to be of insufficient calorie density. Caloric requirements for the military dog have not been determined.

The caloric density of dog food purchased for military use varies from 500 to 2500 calories/lb. The lower figure represents the wet-type canned food. The dry foods in common use range from approximately 1200 to 1600 Kcal/lb. The Federal Specification for dog food does not set limits for caloric density and permits a broad variety of ingredients. The uncertainty of optimal nutritional requirements for working military dogs combined with the spectrum of ingredients permissible under the specification provides little assurance that these dogs are being offered proper rations.

Since the inilitary dog is a valued operational asset, and more and better dogs are to be produced, the need for some definition of their nutritional requirements is obvious.

Commanders in the Republic of Viet Nam (RVN) have too frequently reported that the military dog lacks endurance when employed tactically. Severe weight losses have also been observed. In CONUS, weight loss and lack of endurance have been observed in military Scout Dugs maintained on Standard Item rations, especially in warm, humid climates.

In man it is established that energy requirements are higher in a hot environment. One limited study conducted by the USAF showed that an increase in ambient temperature with or without an increase in relative humidity raises the caloric requirements of the military dog. Weight performance was the essential criterion in that study.

The question has been raised whether current dog rations are adequate for sustained operations in hot, humid environments; primary emphasis appears to be on caloric content. While inadequate caloric intake could well be the limiting factor, other important factors are the animals' state of hydration, their ability to dissipate heat, their electrolyte levels, physical condition and freedom from intercurrent disease.

Whether Standard Item dog food per se influences military dog work performance, health, body weight and appearance should be resolved. Some insight into the caloric requirements of the animals should be developed, especially for work in warm, humid climates.

To begin to investigate some of these points, a representative class of 60 dogs being trained at the Scout Dog School, Fort Benning, Georgia, during July, August, September 1968 was selected for study. The dogs were allotted to 3 subgroups of 20 and each subgroup was fed a different ration: one received a special formulation high in calories and allegedly high in total digestibility (Military Stress Diet-198; MSD-Mark Morris Assoc., Topeka, Kansas); the other groups received one of the Standard Item dry dog food rations, one manufactured by Sturdy Dog Food Co., the other by Gaines (Quaker Oats Co.). Manufacturers' claims are approximately 2500, 1250 and 1600 Kcal/lb. of food respectively for these rations.

The dogs underwent the usual 12-week training. They were offered a fixed amount of food each day (1.9 or 2.2 lbs) and the amount consumed was recorded. They were weighed weekly. Blood levels of electrolytes, enzymes, and other metabolites were determined weekly. Information relative to effects of heat were recorded, i.e. body temperature under various work conditions and at feeding time, amount of water consumed daily, daily ambient temperatures and humidity, assessment of relative hydration of the dogs before and after work by measuring packed erythrocyte volume and serum refractive index.

Weekly fecal examinations for intestinal parasites were performed (esp. hookworms); parasitized animals were treated promptly.

At the end of the 12-week study four male dogs from each group were placed in metabolic cages and a balance study was performed to determine relative digestibility of each of the rations. A dye marker was used, the amount of food eaten for 5 days was recorded and the resultant feces and urine were collected. Aliquots of food and feces were analyzed by bomb calorimetry and proximate analysis.

During the course of the study there was approximately 50% attrition in the dog class, mostly for non-medical rensons (administrative procedures involving dog handlers or dogs not learning fast enough to keep up, etc.). The attrition was uniformly distributed among the 3 test groups and approximately 10 dogs in each group were available for the entire period. Dogs used in the balance study were randomly selected from these.

Data sought were in three general areas: food consumption and weight performance; relative digestibility and nutrient value of the rations; and effects of heat.

RESULTS AND DISCUSSION OF RESULTS:

The field study is complete and only total lipid and cholesterol content of weekly blood samples remain to be determined. These specimens have been frozen since their collection.

Detailed statistical analyses have not been completed.

A. Food Analysis

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Results of the food consumption and relative digestibility portions of the study are summarized in the following table:

	MSD	Sturdy	Gaines
No. dogs completing study	10	8	8
Wgt., Ibs. *	67.5	6 8,2	59.8
% eaten of food offered	77	71	81
Lbs. food eaten per day/dog	1.49	1.53	1.70
Kcal/day consumed	3664	2989	3402
Kcal/day absorbed	3415	2376	273 5
Kcal/b. body wat. consumed	54.6	43.8	56.6
Kcal/lb. body wat. absorbed	50.8	34.8	45.5

Table 1: Food Consumption and Relative Digestibility

*Average body weight of dogs in each group over the duration of the study (74 days).

The values for Kcalories in Table I were derived from calorimetry results of the balance study. Interestingly, the dogs eating Gaines increased their food consumption considerably during the last 4 weeks, achieving an absorbed calorie level comparable to MSD during this period, and raising the average values for the entire period. MSD had a clearly higher digestibility coefficient for all items measured. Analyses of the food and feces are tabulated below:

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Table 2: Food Analysis

Ration	Kcal/lb	%Protein	%Fat	%Carbohydrates	%Moisture	%Ash
MSD	2444.3	27.58	24.39	33.25	9.62	5.16
Sturdy	1956,7	28.15	7.78	48,56	7.97	7.54
Gaines	1928.1	26.03	7.60	51.17	8.76	6.44

Table 3: Feces Analysis

Ration	Kcal/100 gm	%Protein	%Fat	%Carbohydrates	%Moisture	%Ash
MSD	91.05	9.18	1.88	8,94	75.05	4.56
Sturdy	90.00	6.84	1.46	10.88	76.31	4.51
Gaines	85.95	5.55	1.61	12.09	77.00	3.77

Feces from the dogs on MSD were soft and unformed due presumably to the higher fat content and low fiber content (manufacturer's suggestion). The volume was less than the feces from dogs on the other rations.

Comparing absolute quantities of nutrients ingested with those excreted (feces only), the following coefficients of digestibility result (expressed as % of ingested food, except digestible energy is Kcal absorbed/lb of food ingested):

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Ration	Kcal/lb*	Calories	Protein	Fat	CHO	Ash	Dry Matter
MSD	2303.85	94.25	87. 55	97.17	90.25	68. 42	89.93
Sturdy	1543.87	78.90	76.00	81.27	78.57	38.90	73.97
Gaines	1561.55	80.95	80.05	80.15	77.85	45.23	76.37

In Table 4 it is obvious that MSD is better absorbed than Sturdy or Gaines. To express the difference in digestibility between MSD and the other products, the differences for each analysis were calculated and tabulated as percent:

Table 5: Percent Increased Digestibility, MSD over Gaines and Sturdy

Ration	Kcal /16*	Calories	Protein	Fat	CHO	Ash	Dry Matter
MSD over Sturdy	31.52	13.7	13.95	16.57	8.2	18.64	13,98
MSD over Gaines	30, 33	12.8	9.45	18.01	10.7	17.58	13.18

*Kcal absorbed/1b of food eaten.

B. Weight Performance

Weight performance data for the dogs which completed the study reflect the difference in the rations: the dogs on MSD fared considerably better. The MSD fed dogs averaged 5.8 lbs per dog gained over the 12-week period, with 70% of the dogs actually gaining weight. On both the other rations the groups lost weight, averaging 2.5 lbs. per dog, with approximately 70% of the dogs actually losing weight. The weight performance of those dogs which dropped out of the study along the way is similar to that of the ones which remained.

From Table 1, considering absorbed Kcal/lb of body weight, the MSD group absorbed approximately 51 Kcal/lb and the others less than 45.5. The latter groups lost weight, the former gained, indicating a minimum absorbed calorie requirement of approximately 50 Kcal/lb body weight to maintain weight under the environment and work conditions at Ft. Benning during this period. The influence of quality of absorbed nutrients or of the ratio of calories derived from the protein vs. fat vs. carbohydrates cannot be separated in this study.

Food consumption was erratic for all rations throughout the study, most animals appearing to follow cycles of eating more and then less. Food consumption did inversely correlate with ambient temperature at feeding time (the dogs ate less at high temperature) but the importance of this correlation is unknown. MSD was accepted better than the other rations, based on amounts of each consumed during the first 10 days on the test.

A ration of the caloric density and digestibility of MSD has advantages over less efficient rations in addition to the contained nutrients: to provide the necessary absorbed nutrients a considerably smaller weight and volume are required, with the consequent logistic bonus. Financially, this alone might justify use of such a ration. Further, under conditions which may depress appetite (fatigue, heat, other stress) the dog will be more likely to eat sufficient foud to maintain weight,

rather than eat, e.g., half of what is required. (1.4 lbs. of MSD, day would suffice for maintenance of weight of a 55 lb. dog working in warm environment; at least 2.1 lbs. of Sturdy would be required).

We could not detect differences in training efficiency among the dogs, attributable to rations. Many factors contribute to the dogs' response to haining and to their subsequent performance.

Dogs on MSD had sleeker, shiny coats, compared to the dull, dry coats of dogs on the other diets. In clinical veterinary practice such dry coat can be corrected by addition of fats to the dog's ration. Dogs on MSD absorbed nearly four times the fat when compared to dogs on the other rations in this study.

C. Effects of Heat

During July through September the average temperatures during the working day were in the low eighties (°F) with daily maximum temperatures about 10° higher. Relative humidity during the working hours was usually 30–75%.

As part of the Scout Dog Training Program, road marches of 1-5 miles were performed once or twice weekly.

These marches, parts of which are conducted at double time, represent the most strenuous portions of training (except for brief obstacle-course work). The marches contribute to increased physical conditioning and temperature acclimation. Particularly during the initial weeks, body temperatures varied considerably among the dogs while on the marches. From resting temperatures of $100-102^{\circ}F$, essentially all dogs achieved temperature elevations to $104-108^{\circ}F$. Most of these appeared strong and alert, and completed the work without further increase in body temperature. Others however, achieved these temperatures sooner and did not plateau, but continued to increase to $107-108^{\circ}F$. These latter animals became weak, they wobbled and swayed and would have collapsed had they not been rested in shade and "wet down" with whatever water was available (e.g. immersed in streams) to reduce their temperature. After such treatment they could continue, weakly (it is extremely doubtful that these dogs would be efficient scouts under heat stress sufficient to elevate roctal temperature beyond $105^{\circ}F$).

These animals, under the same conditions as their classmates, were unable to dissipate the imposed heat load and thus failed to equilibrate their body temperature at some elevated level, as did their classmates. Their temperatures continued to increase, to the point of transient increase in for most, or to death occasionally.

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While we were unable to identify the factors which determine whether a given animal could equilibrate on a given day, some generalities are applicable. Heat loss under hot conditions (i.e. approaching 950F, ambient) is almost exclusively evaporative, by panting over moist oropharygeal mucosa. As humidity increases, evaporative cooling becomes less efficient. Any factors decreasing an flow over the mucosa (and blood flow through the mucosa) will decrease heat loss. Excitament with barking will interfere, as will jerking against collar or choke chain or presence of a muzzle. Heavy road dust on mucosa may even be influential. The effect of renal function and acid-base control may be involved. As panting increases with increasing body temperature, large excursions in blood pO2, pCO2 and pH occur (the dog is more refractory to alkalemia than man). Alkalosis occurs finally, however, and incapacitation, to death, can result rapidly thereafter. Our general observation was that the dogs we studied were considerably more sensitive to heat and humidity than were the men, and in general were reluctant to leave the shade or to become active on the warm days.

Dehydration was not apparent by comparing serum refractive index (RI) and packed erythrocyte volume (PCV) before and after work daily for several work conditions. (The dogs were allowed to drink water often during the day).

During the first 2-3 weeks, most dogs had to be rested or "wet down." As they became conditioned, approximately 10% still achieved high rectal temperature and were weak and tachypneic. One such dog was monitored continuously with a Yellow Springs rectal thermometer; he collapsed after achieving a temperature of 108°F (the maximum reading on the instrument). Water was not available immediately. The dog died.

The number of dogs which suffer some degree of heat exhaustion is closely related to their working conditions . . . some training instructors are more conservative in introducing their animals to heat stress (shorter, slower marches, more rests), and these groups of dogs had less trouble. Drinking water (canteens, fast-flowing streams) was amply available.

The instructors are aware of a behavioral pattern known as "alerting on shade." During hot periods, many dogs will "alert" toward the shady portion of a sunlit trail... often confusing even experienced handlers. These dogs will pull towards the shade and sit, panting, once there. This occurs while the dog is working, in scouting harness or leash. The frequency of such episodos underscores the potent influence of hyperthermia, and the ingrained behavioral maneuvers to avoid it, despite training to the contrary.

CONCLUSION

While conclusions are premature before all of the data are analyzed, even simple inspection of the tabulated data permits the following:

1. Military Stress Diet-198 (MSD) at approximately 1.5 pounds day per day produced weight gain in 70% of days over a 12-week period.

2. The dry dog foods purchased under federal specifications, Gaines and Sturdy, resulted in weight loss in 70% of dogs over a 12-week period. These dogs ate 1.7 and 1.5 pounds of food dog per day of these rations.

 For all rations, 20-30% of the total quantity offered was left uneaten each day. The initial acceptance (first 10 days) was greater for MSD than for the other rations. Consumption of the Gaines ration increased during the final 4 weeks and during this period the dogs on this ration were consuming almost as many absorbable calories as those on MSD.

4. The digestion coefficients (% absorbed were higher for nutrients in MSD than in the other rations, for all components measured.

5. MSD contains nearly 50% more valories of digestible energy than do the other two diets; dogs on this ration absorbed more calories. Ib. body weight, day (50.8 vs 45.5 for Gaines and 34.8 for Sturdy).

B. A calorie-dense and highly digestible diet such as MSD has the advantage that relatively small amount is required for total nutrient requirements; dogs undergoing stresses which tend to decrease food intake (illness, injur), fatigue, transport, environment extremes) may still get sufficient nutrients to maintain body weight, or recovery of body weight after such stress would be more rapid. Comparable levels of nutrition would require greater volume of other (e.g. current Standard Item) rations and the animals may not eat enough. There is a logistic bonus, especially for overseas shipment.

7. Dog performance in training could not be correlated with diet. Presence of hookworms in some dogs (with frequent examinations and prompt treatment) had no apparent influence on dog weight, food consumption or performance in training.

8. Dogs, even after 12 weeks, are not well adjusted to bot humid conditions and cannot work effectively when rectal temperatures exceed 108°F, if then.

9. Rate of dissipation of body heat appears to be the factor which determines whether a given animal will be able to equilibrate and continue to work under conditions of heat stress.

RECOMMENDATIONS:

1. The Standard Item dry dog food produced by Sturdy Dog Food Co., as represented by the product used in this study is inferior to that produced by Quaker Oat Co. (Gaines). The Sturdy product is not recommended for use as a routine ration for military dogs.

2. A ration of the calorie density and digestibility of MSD-198 is distinctly superior to those currently purchased under the federal specification for dry dog food. Specifications should be developed for procurement of a dog food product having the characteristics of MSD-198, for routine or special-purpose use in military dog feeding.

PUBLICATION:

Bucci, T. J. and Pope, C. R., Letter to the Editor, Jour. Am. Vet. Med. Assn. (in press, June 1969).

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APPENDIX A

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Research in Biomedical Sciences - The objective of this project is to obtain information by the techniques of clinical and basic research on injuries and diseases, except communicable diseases, commonly seen in soldiers, especially in field operations and overseas. The work is divided according to the major medical specialties. a state of the sta

Internal medicine: The objective is to study, by basic research techniques in the laboratory, those diseases of soldiers in the field which are the special province of internal medicine in order to indicate possible approaches to improvements in treatment and prevention. These diseases include diarrhea, hepatitis, anomia, and altered metabolic states in which nutrition plays an efficient or contributory role.

Environmental medicine: The objectives are to obtain basic information on the physiclopical responses of men and animals to climatic changes, especially to heat, cold and high terrestrial altitude, upon which may be based improved procedures for acclimatization, protection and treatment of injury resulting from exposure of soldiers to climatic extremes.

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<u>Bio-Medical Investigations. Military Environmental Medicine</u> - The objective is to develop better methods for the prevention and treatment of diseases produced by the extremes of climate to which a soldier may be exposed.

<u>Bio-Sensor Investigations</u> - The objective of this program is to provide the Army with an improved detector dog capable of tracking, detecting and ush, tunnels, weapons, wines and booby traps--and locating casualties in combat operations. Through a coordinated program of selectivo breeding, evaluation and veterinary research, a superior dog will be developed which has the specific desirable physical, behavioral and sensory characteristics, free of transmissible genetic defects, is responsive to military training, imposes a minimum logistical burden and can perform effectively on or off-leash in all climatic and operational environments. Work at USAMMUL has centered on the influence of the climatic environment on the working detector dog.

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