THE JOURNAL OF NUTRITION

OFFICIAL ORGAN OF THE
AMERICAN INSTITUTE OF NUTRITION

VOLUME 97

JANUARY – APRIL 1969

PUBLISHED MONTHLY BY
THE AMERICAN INSTITUTE OF NUTRITION
BETHESDA, MARYLAND

THE JOURNAL OF NUTRITION ®
® Trade mark registered, U. S. Patent Office

Contents

No. 1 JANUARY 1969

No. 2 FEBRUARY 1969

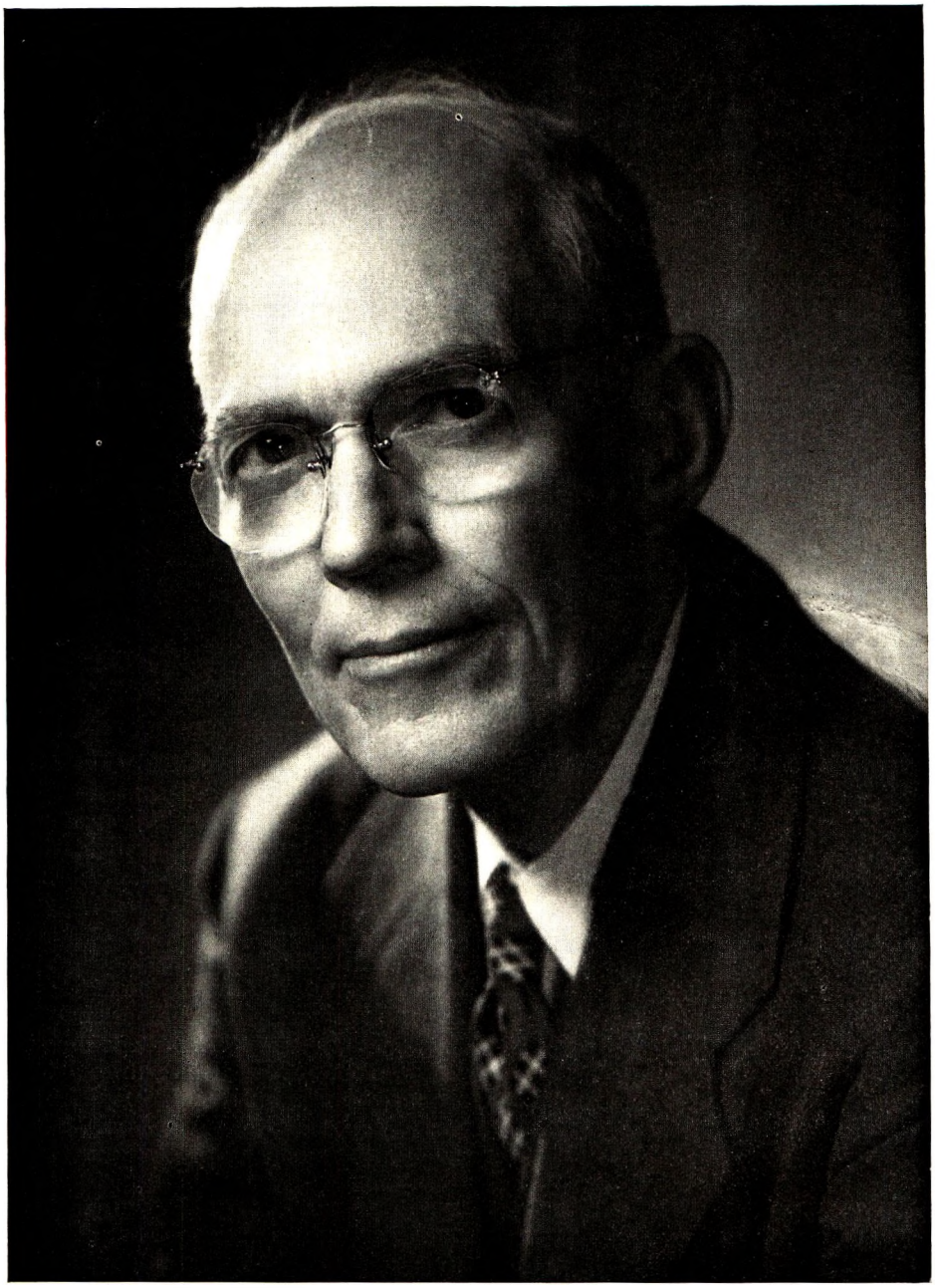
No. 3 MARCH 1969

No. 4 APRIL 1969 (PART I)

No. 4 APRIL 1969 (PART II) — *Supplement 1*

ALBERT GARLAND HOGAN

(1884–1961)



ALBERT GARLAND HOGAN

Albert Garland Hogan

— A Biographical Sketch

(1884 – 1961)

Dr. Albert Garland Hogan spent 35 years of his professional career at the University of Missouri as Professor of Animal Nutrition and Chairman of the Department of Agricultural Chemistry. During this period he made important contributions to basic knowledge in the science of nutrition as well as to the more practical aspects of both human and animal nutrition. He became especially well known to biochemists and nutritional scientists throughout the world for research in several areas of nutrition: definition of the nutritional requirements of different species of animals; separation and identification of members of the vitamin B complex and elucidation of the role of maternal nutrition in the production of irreversible abnormalities in the newborn.

Dr. Hogan was born in Nodaway County near Maryville, Missouri, December 31, 1884. His forebears came to Missouri from England by way of Virginia, Kentucky and Tennessee. His mother was among a group of Kentuckians who came by flatboat to St. Joseph, Missouri, and spread over the rich prairie of northern Missouri in search of homes. There, in Nodaway County, William Carroll Hogan married Belle Ford and to them were born four sons, the second of whom was Albert Garland. Family loyalty, self-discipline and integrity were transmitted from the parents to their sons: the boys walked to country schools, developed strength through outdoor work, and at the same time absorbed classics from the family bookcase.

In the fall of 1903, Dr. Hogan entered the College of Arts and Science at the University of Missouri and in 1907 was awarded the A.B. degree with a major in English and a minor in chemistry. Because of his boyhood experience and the opportunity provided by the University, he continued his education in the agricultural sciences. During the next two years he completed the requirements for the B.S. degree in Agriculture and earned credit toward the Master's degree. After serving on the faculty of Missouri State Normal School at Maryville, Missouri (Northwest Missouri State College) as instructor in chemistry for two years, he returned to the University of Missouri for further graduate study and received the M.A. degree in June, 1912. The research for the Master's thesis involved the chemical analysis of a full-term bovine fetus. In the fall of 1912, he enrolled in the graduate school of Yale University where he was a student of Dr. Lafayette B. Mendel and an assistant in chemistry in the Sheffield School of Science. He obtained the doctoral degree in physiological chemistry in 1914. Four papers were published as the result of research accomplishments while he was a student at Yale. One was a new method of preparing xanthine and three were studies related to carbohydrate metabolism.

From 1914 to 1917, he was an Assistant and Associate Professor at Kansas State College where he taught chemistry and investigated the nutritive value of corn and kaffir proteins. When the United States entered World War I in 1917 he was commissioned an officer in the Sanitary Corps of the 81st Division, U.S. Army. He served in France with the American Expeditionary Forces through the Meuse-Argonne drive and was promoted from Lieutenant to Captain. He was discharged from military duty in 1919 and accepted a position as Professor of Physiological Chemistry at Alabama University Medical School. During this period he met Theodosia Catherine Cobbs and they married in Mobile, June 23, 1920. Three daughters were born to this union, Catherine Hogan Minor, Amelia Hogan Graves and Madge Hogan McKercher. In the fall of 1920, he was called

to the University of Missouri as Professor of Animal Nutrition. Three years later he was given the additional responsibility of chairmanship of the Department of Agricultural Chemistry. He held both positions from 1923 until his retirement in 1955.

The significance of Dr. Hogan's contributions to the science of nutrition and the recognition of his distinguished career as a teacher and researcher are shown most clearly by the awards which he received. These were the Mead Johnson Award in 1944 for research related to the vitamins; the Morrison Award in 1951 for work in animal nutrition; the Borden Award in Nutrition in 1955; and the Osborne and Mendel Award in 1956. The citation of the latter award read, "for his development of synthetic rations for use in nutritional studies and for his original investigation in the field of biochemistry and nutrition which made him one of the greatest contributors to the development of our present knowledge of animal nutrition."

Dr. Hogan was a member of several scientific and professional societies including Gamma Sigma Delta, Alpha Chi Sigma, Sigma Xi, American Chemical Society, American Association for the Advancement of Science, American Society of Biological Chemists, Society of Experimental Biology and Medicine, American Society of Animal Science, Poultry Science Association and American Institute of Nutrition. He served on the Editorial Board of the *Journal of Nutrition* from 1937 to 1942, and as Vice-President, 1940–1941, and President, 1941–1942, of the American Institute of Nutrition. He was made a Fellow of the Institute in 1959 and was a life member of the Poultry Science Association.

As professor of Animal Nutrition, Dr. Hogan taught many undergraduate students the basic principles of nutrition and supervised the research and preparation of the theses of 45 Master's and 20 Ph.D. candidates. Many of the students who received the Master's degree continued to make important contributions in industrial and research institutions and others pursued graduate training at other institutions. Those who received the doctorate degree achieved prominence as directors of nutrition departments in industry and as professors and administrators in universities across the country. These students have continued to spread and advance the knowledge of nutrition by their teaching and research activities.

One hundred and sixty-nine papers and abstracts were published in 27 scientific journals as the result of the research carried out under Dr. Hogan's supervision. These papers covered many aspects of nutrition in a variety of animals, but there was a common thread which gave them a unity of purpose. His guiding goal was to define the nutrient requirements for the complete life cycle. During the early part of his career he was concerned primarily with the fundamental aspects of nutrition in domestic animals, but later his interest was directed toward problems of public health significance. The qualities that most clearly characterize Dr. Hogan's research papers were their originality, clarity and precision. He always worked at the forefront of the research endeavor of his day.

Early in his career at Missouri he wrote a chapter in *Growth*, a book published by the University of Missouri Chapter of Sigma Xi, and over the years contributed numerous original and review articles to the Missouri Agricultural Experiment Station Research Bulletins. Reviews of wider distribution include: Physiology and Pathology of Riboflavin published in the symposium monograph on Vitamins under the auspices of the American Medical Association; The Vitamin Requirement of Poultry in Nutrition Abstracts and Reviews; Nutrition in Annual Reviews of Biochemistry; and the Newer Hematopoietic Factors of the Vitamin B Complex in Vitamins and Hormones.

As mentioned above, Dr. Hogan's outstanding contributions were in the areas of basic nutrition, but his research interest included practical problems of feeding experimental and domestic animals. His early publications stemmed from investigations related to the nutritive value of various supplements in rations for domestic animals. These studies were carried out chiefly with rations containing natural feedstuffs. His objective was to achieve optimal growth and reproduction during complete life cycles of different species, and he soon became convinced that it would be necessary to develop diets com-

posed of chemically defined ingredients. He began the difficult task with the premise that the basic ingredients, such as carbohydrates, protein, fat and minerals, must be free of contaminating impurity. Consequently, much effort was spent in removing any possible impurities from commercial products which normally are considered relatively pure. For example, corn starch and cane sugar were extracted repeatedly with different solvents and casein was reprecipitated and thoroughly washed. This treatment was frequently followed by exhaustive extraction with hot ethanol and with ethyl ether.

All of this laborious purification was carried out in the laboratory and much of his success hinged on the innovations he conceived. Of course, animals fed the highly purified ingredients failed to grow because vitamins were missing. These had to be supplied as concentrates from wheat germ, rice polishings, yeast or liver. Dr. Hogan and his students spent many long hours preparing these vitamin concentrates during the years before vitamins were commercially available. Beef liver and yeast were main sources of unrecognized vitamins during the 1930's and 1940's. Large quantities of beef liver and yeast were extracted and processed in the departmental laboratories. The extracts were tested in animals and active fractions were subjected to more refined procedures such as adsorption, extraction and precipitation in an attempt to obtain the activity in a highly concentrated form.

His publications during this period cover the methods used to concentrate and assay various vitamins associated with the vitamin B complex, as well as their value for growth and reproduction in experimental animals. Probably the most significant observation made during the course of these studies was the fact that baby chicks fed a purified diet containing all of the vitamins known at that time developed a macrocytic anemia. Dr. Hogan was quick to realize the medical significance of this observation and proceeded to prove beyond doubt that the anemia resulted from a deficiency of an unrecognized vitamin. He named it vitamin B_c, a B vitamin required by the chick, and used the cure of anemia as an assay method. During this period there was a cooperative arrangement with the Research Laboratories of Parke, Davis and Company which resulted eventually in the isolation of crystalline vitamin B_c. This vitamin, now known as folic acid or folacin, was under investigation in several laboratories at that time and was given several different names before its structure was finally elucidated. Although vitamin B_c did not prove to be the antipernicious anemia factor as Dr. Hogan had hoped in the early days, it has proved to be of great value in human health.

Even before his interest in folic acid anemia, Dr. Hogan had investigated an anemia in rats caused by feeding deaminized casein. Casein was treated with nitrous acid in an attempt to decrease its lysine content. Rats fed deaminized casein as the source of protein grew poorly and developed a severe anemia which was not prevented by casein or a hydrolyzate of casein. Lysine was the protective amino acid but it turned out that a lysine antagonist had been produced by the deamination process. Although this project was not in the main stream of his research activities, it does reflect his long standing interest in lysine as the limiting amino acid of corn. This interest developed during his first position at Kansas State College. In other studies purified diets based on casein proved to be unsatisfactory for chicks. Although this was first thought to be due to an unrecognized vitamin deficiency, he later found that the poor growth was due to an amino acid imbalance which was corrected by arginine and glycine.

Another interest that deviated somewhat from his specialty in nutrition was that of blood coagulation and hemostasis. In 1941 he was the senior author of the first research report to describe a hemophilia-like disease in swine. These pigs observed as a part of a breeding study at the Missouri Experiment Station, provided an experimental model for research on an inherited biochemical abnormality that parallels a condition in man that has been known for several decades. The hemophilia-like condition was shown to be a deficiency of a blood protein associated with thrombin generation. The deficient factor appears to be identical with the human antihemophilia factor (AHF) or factor VIII. The bleeder

swine research was supported by the first departmental grant from outside the College and was later generously supported by outside grants.

Dr. Hogan was always interested in the nutritional problems faced by the farmers of the State. He worked closely with the diagnostic veterinarian in the School of Veterinary Medicine and took advantage of the opportunity to investigate diseases, especially in poultry, which were not caused by infectious agents. Frequently, he was able to demonstrate that the trouble was due to a nutrient deficiency in the diet. The first case of curled-toe paralysis was seen in the laboratory when baby chicks were brought to the laboratory by a woman who raised a few chickens for family consumption. Administration of crystalline riboflavin to the chicks cured the symptoms dramatically within twenty-four hours.

Some of his most important scientific contributions may be summarized as follows: He showed that the original vitamin B_2 was a mixture. The pellagra-like syndrome described by Goldberg and Wheeler was cured or prevented by riboflavin alone and it was concluded that vitamin B_2 or G was identical with riboflavin. He discovered vitamin B_c or folic acid and announced its existence in 1939. Assay methods were developed which led to its isolation and identification. His work allowed the development of purified diets which supported a complete life cycle; second generation rats, chickens and swine grew to sexual maturity and produced normal young. He clearly showed a relationship between nutrition and congenital malformations. Deficiencies of folic acid and vitamin B_{12} in the maternal diet gave rise to abnormalities in newborn rats such as hydrocephalus and cleft palate. He demonstrated the importance of potassium and magnesium in preventing a condition in experimental animals which was similar to arthritis in man. These and many other significant contributions to the science of nutrition have been widely recognized as benefiting not only livestock production but human health as well. For the producers of meat, milk, poultry and eggs his contributions have lowered production costs and improved the quality of the products. They have benefited producer, processer and consumer and have added millions of dollars to the national economy.

Administrators at the University sought and used Dr. Hogan's advice because of their high regard for his personal integrity and sound judgment. He served as a consultant to industry and government on many occasions and his opinion was held with deep respect by both scientists and laymen with whom he came in contact. His ability to project his own high principles logically and succinctly earned him the highest esteem.

Dr. Hogan said of himself that, if he had accomplished anything, it was because he had worked harder in a relaxed way. He felt that his main ability was in adapting the use of equipment and inventing ways to accomplish an objective with the meager funds which were available. His budget did not allow the purchase of every modern piece of equipment that became available and he realized that this was not essential to success. Frequently, he remarked that his work was the most interesting thing that he knew about and he needed no hobby. He was as methodical in his personal habits as in his work and a neighbor remarked that his clock could be set by the time Dr. Hogan went to his office. He possessed remarkable poise and dignity in personal relationships and yet approached problems dynamically. Graduate students and staff always looked forward to the social events in the Hogan home.

His understanding of and patience with students, his consideration for his associates, and his professional dedication created an atmosphere which stimulated students and associates to perform to their best ability. His high standards, personal example and interest in students and staff fostered a unity of purpose and a desire to excel in order to justify and reward his confidence.

Despite his zest for hard work, Dr. Hogan found time for outside activities. He gave much time to the community, university, and his church. He participated frequently in social affairs and enjoyed them immensely. He could talk on almost any subject but he did not give a positive opinion unless he had given serious thought to the subject under discussion. He played

golf well and frequently won high scores at an occasional bridge game. He had a good sense of humor and always enjoyed a good joke especially when it was on himself. The informal gatherings of former students and staff were always a highlight of the annual Federation, Animal Science and Poultry Science meetings. The relating of humorous instances that occurred at the laboratory as well as the more serious exchange of ideas about current problems in nutrition produced a closeness that was appreciated by everyone.

At the time of Dr. Hogan's retirement a large number of faculty, friends and students attended a dinner to honor his 35 years' service to the University. At this dinner a bound volume of 110 letters of appreciation was presented to him and a Hogan Fellowship in Nutrition and portrait of him were presented to the University. Dr. Hogan's continued interest in nutrition after his retirement was demonstrated by his continued punctual and daily appearance at his office in Schweitzer Hall and his attendance at national and international meetings in nutrition and related fields.

During the last five years after retirement Dr. Hogan served as a consultant for the National Live Stock and Meat Board. It was from a trip in the interest of the Meat Board's research program that he had to return home because of an illness which ended in his death January 25, 1961.

It was only fitting that friends established in his honor the Hogan Memorial Library for the benefit of a new generation of nutritionists.

Grateful acknowledgment is made to Mrs. A. G. Hogan, Dr. Merle Muhrer, Dr. Boyd O'Dell and Dr. James Savage for providing material for this paper and for reading the manuscript.

L. R. Richardson, Ph.D.,
5 Kathryn Avenue,
Florence, Kentucky 41042

Effect of Vitamin A Deficiency on Mitochondrial Lipids in Rat Liver [1]

G. VAUGHAN MITCHELL, C. R. SEWARD AND M. R. SPIVEY FOX
Division of Nutrition, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C.

ABSTRACT A study was made to determine the effect of vitamin A deficiency on mitochondrial lipids in the livers of young rats. The purified basal diet, which contained 18% casein and 10% stripped lard, was fed with no added vitamin A, or with 5 mg vitamin A acetate/kg. Some of the vitamin A-supplemented rats were pair-fed with deficient rats to obviate the effect of inanition. After 42 to 60 days, weight loss characteristic of vitamin A deficiency occurred; animals were then killed and liver mitochondrial lipids were analyzed. Cholesterol and triglycerides from vitamin A-deficient animals were significantly higher than those of corresponding pair-fed animals and controls fed ad libitum. The deficient rats also showed a significant decrease in liver mitochondrial lipid phosphorus and total lipid. Mitochondrial lipids from the pair-fed controls were significantly higher in arachidonic and linoleic acids when compared with deficient rats or with controls whose food intake was not restricted. Vitamin deficient rats and pairfed controls had significantly less palmitoleic acid in their mitochondrial lipids than did control rats fed ad libitum.

Since the discovery by Wald in 1934 (1) that vitamin A is involved in the visual cycle, much effort has been directed toward elucidating its action at the molecular level. Evidence from several types of investigation supports the hypothesis that vitamin A performs a metabolically significant role in maintaining functional integrity of subcellular membranes by physical and chemical means. Roels et al. (2) demonstrated that the incorporation in vitro of ^{14}C-labeled amino acids into diaphragm protein was higher for rats receiving a diet deficient in vitamin A than for the pair-fed controls. These authors explained their results in terms of an effect of vitamin A on membrane permeability and stability. Several other recent reports (3–7) also indicate that vitamin A may have a role in membrane permeability and stability. Deluca et al. (3) interpreted the high oxygen uptake by liver homogenates from vitamin A-deficient rats as a possible indication of a general membrane disturbance. Results supporting this view were published by Seward et al. (4, 5) who suggested that the role of vitamin A in controlling oxidative phosphorylation efficiencies may be mediated through its ability to affect mitochondrial membrane permeability. Lucy and Dingle (6, 7) have extensively investigated the effect of vitamin A on membranes of mitochondria, lysosomes and erythrocytes and have suggested that vitamin A is necessary for the stabilization of membranes by acting as a cross-linking agent between lipid and protein.

Because lipids are known to be important in mitochondrial membranes and because vitamin A deficiency alters mitochondrial function, a study of the effects of vitamin A deficiency upon mitochondrial lipid classes and fatty acid profiles should be of value.

MATERIALS AND METHODS

Male weanling rats of the Holtzman strain,[2] 21 days old and weighing from 40 to 50 g, were divided into groups of 10 or 20 for these studies. The animals were housed individually in suspended stainless steel cages at a constant room temperature of 23 ± 1°. A purified 18% casein diet (4) with vitamin A omitted was fed to produce vitamin A deficiency; all other animals received the same diet supplemented with 5 mg vitamin A acetate/kg diet and were designated as controls. This level of vitamin A maintains normal growth but is

Received for publication July 26, 1968.

[1] The experimental data in this paper are taken from a thesis submitted by G. Vaughan Mitchell in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry, George Washington University, Washington, D. C.

[2] Holtzman Company, Animal Breeders, 421 Holtzman Road, Madison, Wis.

not toxic. Diets were prepared weekly and refrigerated until fed. Stripped lard at a level of 10% served as the only source of fat in the diet. In experiment 1, food was available ad libitum. The vitamin A-deficient rats, together with controls, were killed when the deficient rats had lost weight for at least 4 consecutive days. The onset of body weight loss occurred within 42 to 50 days. In experiment 2, the food intake of each rat receiving vitamin A was restricted to that of a littermate rat of the same starting weight but fed the deficient diet. Experiment 2 was begun 1 month after the end of experiment 1. Vitamin A deficiency was determined by weight loss for 4 days, as in experiment 1. The onset of body weight loss occurred after 52 to 60 days.

After the rats were killed, their livers were quickly excised and liver mitochondria were prepared from a 10% (w/v) homogenate in a medium of 0.25 M sucrose according to the centrifugation method of Weinback (8). Nitrogen was determined by the micro-Kjeldahl technique and vitamin A by the method of Ames et al. (9).

Lipid analyses. Mitochondrial lipids were extracted with methanol–chloroform as described by Folch et al. (10) and the extracts from two rats were pooled to provide sufficient lipids for the analyses. Total lipid content was determined gravimetrically after evaporation of solvent. Total cholesterol was determined by a modification of the Schoenheimer-Sperry method (11) and lipid phosphorus by the method of Bartlett (12).

Triglycerides were first separated with thin-layer chromatography by the method of Schlierf and Wood (13) and were eluted with chloroform. Glycerol was then determined by the method of Neish (14), using triolein as a standard.

Gas-liquid chromatography. Mitochondrial lipids were interesterified by the method of Stoffel et al. (15). The fatty acid methyl esters were separated by gas-liquid chromatography on a gas chromatograph [3] equipped with a β-ionization detector, using argon as the carrier gas. The stationary phase was 14% ethylene glycol succinate polyester coated on 80–100 mesh Gas Chrom P [4] packed into a 183 cm by 0.4 cm (inner diameter) glass column. The column was operated at 190° and at an inlet pressure of 14 psi. Quantitative results, obtained by triangulation, are expressed in terms of area percent. The individual fatty acids determined were the same as those present in the stripped lard added to the diet. They were identified by using equivalent chain-length values derived from standards.

RESULTS

Growth. Growth curves are shown in figure 1. In both experiments, regardless of pair-feeding or feeding ad libitum, the growth was greater for the control rats than for the deficient rats. Vitamin A-deficient rats grew at the same rate as the supplemented animals for 3 weeks in experiment 1 and for 4 weeks in experiment 2. When control rats were pair-fed with deficient rats (3 versus 4) the control gained significantly more weight between weeks 4 and 7 than the deficient rat. The weight gains of pair-fed controls and controls fed ad libitum were 21 g/week and 39 g/week, respectively.

Content of liver mitochondrial lipids. The amounts of vitamin A in the mitochondria from livers of control and vitamin A-deficient rats in experiment 1 were 502

[3] Model 10 Gas Chromatograph, Barber-Colman Company, Rockford, Ill.

[4] Pretested packing and methyl ester standards were obtained from Applied Science Laboratories, State College, Pa.

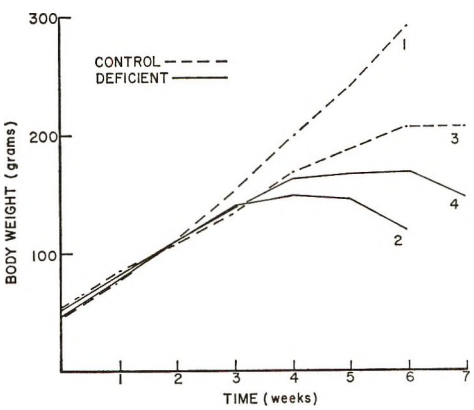


Fig. 1 Effect of vitamin A deficiency on the growth of male weanling rats. In experiment 1 (curves 1 and 2), rats were fed ad libitum and in experiment 2 (curves 3 and 4), vitamin A-supplemented rats were pair-fed to deficient rats.

μg/g protein and nondetectable, respectively. Concentrations below 5 μg vitamin A/g liver are not detected by this method (9). Table 1 shows the effects of vitamin A deficiency on various liver mitochondrial constituents. In experiment 1, the contents of total lipid and of lipid phosphorus were significantly lower in liver mitochondria from rats fed a vitamin A-deficient diet as compared with controls. The cholesterol and triglyceride contents of mitochondria from vitamin A-deficient rats, however, were markedly higher than those from rats receiving dietary supplements of vitamin A acetate.

To eliminate or minimize any possible effects on inanition, which usually occurs in only the later stages of vitamin A deficiency, a group of control rats was pair-fed individually against animals ingesting the vitamin A-deficient diet. The results from the pair-fed group (table 1, exp. 2) were similar to those of rats fed ad libitum (exp. 1). As in the case of liver mitochondria from the animals fed ad libitum, the total lipids and lipid phosphorus were significantly decreased whereas the cholesterol and triglyceride were markedly elevated in the vitamin A-deficient rats.

Fatty acid composition of total mitochondrial lipid. The mean values for the fatty acids of the total mitochondrial lipids from vitamin A-deficient and control rats are shown in table 2. Minor fatty acids (less than 1%) are not shown. The level of palmitoleic acid ($C_{16:1}$) in mitochondrial lipid from rats ingesting the vitamin A-deficient diet showed a significant decrease compared with their controls (exp. 1), but no other significant difference was noted. In experiment 2, in which the fatty acid composition of the mitochondrial lipid of vitamin A-deficient rats was compared with pair-fed controls, the changes were more pronounced. Linoleic ($C_{18:2}$) and arachidonic ($C_{20:4}$) acids were significantly decreased by 43 and 31%, respectively, in the vitamin A-deficient rats.

DISCUSSION

In these studies the concentrations of several categories of lipids in rat liver mitochondria were influenced by vitamin A deficiency. All values for vitamin A-deficient animals in the two experiments were similar except for total cholesterol, which was higher in experiment 2. A comparison of both the animals fed ad libitum and pair-fed controls with corresponding vitamin A-deficient rats showed that cholesterol levels in the mitochondria were significantly increased as a result of the deficiency. This relationship needs further investigation because of the variability in the deficient animals between experiments; however, this trend in the liver mitochondria of the deficient rat is in agreement with the data of Diplock et al. (16), who found that vitamin A deficiency caused an increase in total liver cholesterol when compared with pair-fed and control rats fed ad libitum.

The amounts of total lipid phosphorus and total lipid per unit of mitochondrial protein was significantly decreased in vitamin A-deficient rats. Vakil et al. (17) reported that the concentration of total phospholipids in the liver of male rats de-

TABLE 1

Effect of vitamin A deficiency on lipid classes of rat liver mitochondria [1]

Group	Total lipid	Total cholesterol	Lipid phosphorus	Triglyceride
	mg/g protein	*mg/g protein*	*mg/g protein*	*mg/g protein*
		Exp. 1 (ad libitum)		
Deficient	186 ± 4.0 *	5.6 ± 0.39 **	4.8 ± 0.07 **	10.2 ± 0.96 *
Control [2]	211 ± 5.6	3.8 ± 0.40	5.7 ± 0.11	7.9 ± 0.55
		Exp. 2 (pair-fed)		
Deficient	205 ± 13.7 **	7.1 ± 0.03 **	5.1 ± 0.18 **	10.3 ± 0.30 *
Control [2]	247 ± 6.4	5.4 ± 0.50	6.6 ± 0.45	7.1 ± 1.26

[1] Mean ± SE; 20 rats/group.
[2] Animals supplemented with vitamin A are designated as controls.
* Significantly different from control ($P \leq 0.05$).
** Significantly different from control ($P \leq 0.01$).

ficient in vitamin A was lower than in controls receiving 17.2 μg vitamin A acetate daily.

One reasonable mechanism can be proposed to explain the observed changes in lipid classes resulting from vitamin A deficiency. Vitamin A may be an integral structural component of the cellular and subcellular membrane. A shift in the relative concentration of vitamin A in the membranes may cause changes in permeability and stability.

The relative concentrations of individual fatty acids in the mitochondrial lipids paralleled, in general, those of the dietary lard (table 2). Other workers have shown that the fatty acid content of liver mitochondria can be markedly influenced by the fatty acids in the diet. For example, Bartley and Getz (18) found that liver mitochondrial lipids contained 5.1% decosahexaenoic acid. We found no decosahexaenoic acid in rat liver mitochondrial lipids and there was none in our diets. Witting et al. (19) reported that rats fed cod liver oil could incorporate the decosahexaenoic acid from the diet into liver mitochondrial lipids.

The percentage distributions of the fatty acids were similar for the vitamin A-deficient rats in both experiments. In experiment 1, the value for palmitoleic acid was significantly lower than that for the controls. In experiment 2, however, the palmitoleic acid value was low, not only for the vitamin A-deficient rats, but also for the controls whose food intake was restricted. It must be concluded that this effect was due to dietary restriction and not to vitamin A deficiency. No other significant differences between vitamin A-deficient and control animals were observed in experiment 1.

The percentages of linoleic and arachidonic acids were elevated in pair-fed control rats (exp. 2) when their values were compared with those of vitamin A-deficient rats, which can be considered to have self-restricted food intake, or the control rats eating ad libitum in experiment 1 ($P < 0.01$). This effect of food restriction must have developed fairly rapidly since food intake was not severely reduced until the last few days of the experiment, as indicated by the growth curves in figure 1. The data suggest that vitamin A may influence the metabolism of linoleic and arachidonic acids under conditions of restricted food intake since the proportions of these acids did not increase in the deficient animals. There was a trend toward higher levels of saturated fatty acids in the liver mitochondria of vitamin A-deficient animals as compared with pair-fed controls (exp. 2); however, the differences for palmitic and stearic acids were not statistically significant.

ACKNOWLEDGMENTS

The authors thank Dr. A. J. Sheppard and Mr. A. R. Prosser for their advice on experimental procedures related to gas-liquid chromatography and Mr. H. Valentine for his technical assistance.

TABLE 2

Effect of vitamin A deficiency on fatty acid composition of total mitochondrial lipids in rat liver and relation to dietary fat [1]

Fatty acids	Stripped lard	Exp. 1 (ad libitum)		Exp. 2 (pair-fed)	
		Deficient	Control [2]	Deficient	Control [2]
	%	%	%	%	%
$C_{14:0}$	1.6	—	—	—	—
$C_{16:0}$	36.1	30.7 ± 0.52	29.3 ± 0.77	30.1 ± 1.72	25.0 ± 2.50
$C_{16:1}$	2.6	3.4 ± 0.80 **	6.2 ± 0.35	3.9 ± 0.58	3.7 ± 0.55
$C_{18:0}$	11.6	16.7 ± 1.12	17.0 ± 1.12	19.6 ± 1.60	14.5 ± 1.37
$C_{18:1}$	40.5	26.5 ± 1.57	25.5 ± 0.98	23.8 ± 0.50	21.6 ± 1.45
$C_{18:2}$	7.6	6.2 ± 0.72	6.0 ± 0.22	6.6 ± 0.30 **	11.5 ± 1.14
$C_{20:3}$	—	2.5 ± 0.33	2.4 ± 0.14	2.0 ± 0.94	2.5 ± 0.25
$C_{20:4}$	—	13.6 ± 0.57	13.5 ± 1.75	14.0 ± 1.06 **	21.1 ± 0.95

[1] Mean ± SE; 10 rats/group.
[2] Animals supplemented with vitamin A are designated as controls.
** Significantly different from control ($P \leq 0.01$).

LITERATURE CITED

1. Wald, G. 1934 Carotenoids and the vitamin A cycle in vision. Nature, *134:* 55.
2. Roels, O. A., M. Trout, A. Guha, U. K. Vakil and K. Joseph 1964 Effect of dietary α-tocopherol and protein metabolism in vitamin A-deficient rats. J. Nutr., *84:* 161.
3. Deluca, H. J., M. R. Manatt, N. Madsen and E. B. Olson 1963 Action of vitamin A on liver homogenate oxidation of tricarboxylic acid cycle intermediates. J. Nutr., *81:* 383.
4. Seward, C. R., G. Vaughan and E. L. Hove 1964 Respiratory activities of hypo- and hyper-vitaminotic-A rat liver homogenates. Proc. Soc. Exp. Biol. Med., *117:* 477.
5. Seward, C. R., G. Vaughan and E. L. Hove 1965 Effect of vitamin A deficiency or excess on the oxidative phosphorylation by rat liver mitochondria. J. Biol. Chem., *241:* 1229.
6. Dingle, J. T., and J. A. Lucy 1962 Studies on the mode of action of excess of vitamin A. Biochem. J., *84:* 611.
7. Lucy, J. A., and J. T. Dingle 1962 Vitamin A and membrane systems. Biochem. J., *84:* 76P.
8. Weinback, E. C. 1961 A procedure for isolating stable mitochondria from rat liver and kidney. Anal. Biochem., 2: 335.
9. Ames, S. R., H. A. Risley and P. L. Harris 1954 Simplified procedure for extraction and determination of vitamin A in liver. Anal. Chem., 26: 1378.
10. Folch, J., M. Lees and G. H. Sloane-Stanley 1957 A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226: 497.
11. Sperry, M. W., and M. Webb 1950 A revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
12. Bartlett, G. R. 1959 Phosphorus assay in column chromatography. J. Biol. Chem., 233: 466.
13. Schlierf, G., and P. Wood 1965 Quantitative determination of plasma free fatty acids and triglycerides by thin-layer chromatography. J. Lipid Res., 6: 317.
14. Neish, J. 1950 Determination of glycerol and related compounds in fermentation solution by partition chromatography. Can. J. Res., 28: 83.
15. Stoffel, W., F. Chu and E. H. Ahrens, Jr. 1959 Analysis of long chain fatty acids by gas-liquid chromatography: micromethod for preparation of methyl esters. Anal. Chem., *31:* 307.
16. Diplock, A. T., J. Green and J. Bunyan 1965 Vitamin A and isoprenoid synthesis in the rat. Biochem. J., 95: 138.
17. Vakil, U. K., O. A. Roels and M. Trout 1964 Storage and transport of vitamin A in relation to protein intake. Brit. J. Nutr., *18:* 217.
18. Bartley, W., and G. S. Getz 1961 The intracellular distribution of fatty acids in rat liver. Biochem. J., 78: 307.
19. Witting, L. A., C. C. Harvey, B. Century and M. K. Horwitt 1961 Dietary alterations of fatty acids of erythrocytes and mitochondria of brain and liver. J. Lipid Res., 2: 412.

Apparent Carotenoid Increases in the Digestive Tract of Beef Cattle

R. ALMENDINGER AND F. C. HINDS
Ruminant Division, Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT Carotenoid determinations were made on haylage, hay and the feces of cattle fed these roughages. Excretion of β-carotene and other carotenoids was much greater than the amount contained in the haylage and was about equal to that contained in the hay. Apparent increases in β-carotene and total carotenoid were observed in haylage samples incubated with hydrochloric acid when concentrations approximated the conditions found in the abomasum, but this did not account for the total amount of pigment in the feces. This apparent increase was probably due to a releasing of residual carotenoids not extracted by the usual procedures.

In recent studies on the growth and digestibility performance of beef cattle fed hay and haylage,[1] it was found that β-carotene seemingly was excreted in greater amounts than ingested by cattle fed haylage; with hay, the carotene was excreted in quantities more nearly equal to those consumed. Liver stores of vitamin A declined with haylage, although the amount of β-carotene consumed was 6 to 39 mg/lb. of feed, a level well above 0.7 mg/lb. of feed, the published requirement of the National Research Council (1). These facts seemed to suggest that not all of the carotene in the haylage is available to either the animal or the assay procedures. Therefore, a study of the effects of the digestive tract on the measured carotene in the haylage was undertaken. This paper reports the effects of the simulated conditions found in the abomasum on the apparent β-carotene content of haylage.

EXPERIMENTAL

For the experiments in vivo, growing cattle of various breeds and both sexes were fed ad libitum a diet of hay or haylage with a trace-mineralized salt block,[2] available free-choice for 108 and 87 days in the years 1965 and 1966, respectively. A 3-lb. grain supplement (6 parts, oats; 3 parts, corn; 1 part, wheat bran; and 1 part, linseed-meal pellets by weight) was included in the diet daily up to 1 week before sampling for carotenoid analysis.

The hay and haylage were made from the second cutting of a legume-grass stand with alfalfa predominating. Red clover, timothy and orchardgrass comprised less than one-quarter of the dry matter. The alfalfa was in three-quarter bloom and the other species were in the seed stage. After mowing, conditioning, windrowing and wilting, half of the field was chopped for haylage and stored in a glass-lined, gastight structure. The rest of the field was allowed to dry and was baled. For feeding purposes, the hay was chopped using the same harvester as for the haylage.

Following a 2-week preliminary period, total feed input and fecal output records were kept for 8 consecutive days once each year. Fecal "grab" samples were taken and immediately frozen in tightly closed plastic bags. Corresponding feed samples were also frozen and carotenoid analysis was done on each individual sample.

For carotenoid analysis, 10 to 20 g of sample were homogenized several times in a mixer[3] at high speed with 100 ml each of the following solvents: acetone, once; acetone-Skellysolve-B, 55–45, three times; Skellysolve-B, three times; and acetone, until colorless. The Skellysolve-B was washed three times with 300-ml aliquots of water and then saponified under nitrogen for 16 hours at room temperature, in the dark, by gently shaking with a fresh solution of 90 ml 95% ethanol saturated

Received for publication June 24, 1968.

[1] Hinds, F. C., P. E. Lamb, B. E. Bremer, R. Almendinger and U. S. Garrigus 1967 Hay and haylage for beef cattle. J. Anim. Sci., 26: 1493 (abstract).

[2] Diamond Crystal Salt Company, St. Clair, Mchigan.

[3] Omni-Mixer homogenizer, Ivan Sorvall Incorporated, Norwalk, Connecticut.

with KOH. The KOH was washed from the Skellysolve-B by a 90-ml aliquot of water, then with several 500-ml portions of water until the water was colorless when tested with phenolphthalein. After all traces of water were removed with Na_2SO_4, chromatography was carried out by two methods. For individual characterization of all the carotenoids present, a 5% water-deactivated alumina column (30 by 1 cm) was used with a 1-cm layer of Na_2SO_4 on top. Stepwise gradient elution with the following solvents was employed: Skellysolve-B, diethyl ether, acetone and methanol. Bands that were not homogeneous were rechromatographed on activated alumina (40 by 1 cm) with the same solvents. Characterization and identification of the compounds were based on their position on the chromatogram, the absorption spectra in hexane, the epoxide test of Curl and Bailey (2) and the 30-minute isomerization test of Jungalwala and Cama (3).

The less time-consuming AOAC (4) chromatographic procedure was employed after the same extraction and saponification in the initial study (1965) and in the in vitro studies for estimation of β-carotene and the total carotenoid content.

Peptic digests were run in vitro on 1-g freeze-dried samples at 40°, with CO_2 or nitrogen bubbling through the system, using a fresh solution of 100 ml 0.2% pepsin in 0.075 N HCl. Some samples were previously extracted with diethyl ether or acetone–Skellysolve-B, or both, until colorless. Postincubation extraction and chromatography were carried out as above. Controls with hydrochloric acid or water were also run at various temperatures and pHs using a 0.5 M Tris-HCl buffer to maintain the pH above 7. Water from a deionizer was used for the pH 5 incubation since this is the routine pH for the laboratory's supply and a buffer was not necessary to maintain pH throughout the incubation.

Extracting, by refluxing a 1-g sample with 50 ml 95% ethanol, or 45 ml 95% ethanol and 5 ml 60% KOH for 30 minutes in the dark, also was investigated as a method of increasing the carotenoids extracted from the haylage.

RESULTS

From the β-carotene analysis of samples collected in 1965 it was calculated that steers fed hay excreted from 83.4 to 90.1% of the carotene found in the feed whereas animals fed haylage excreted from 172 to 184%.

To investigate the possibility of noncarotene pigments being present in the AOAC assay, and to determine if the β-carotene might be increasing at the expense of other carotenoids, detailed assays for individual carotenoids were used on samples collected in 1966 and the results are shown in table 1. The hydroxylated compounds were grouped together as they all showed the same trend of an apparent higher amount excreted than eaten and their identification could not be positive since there were no known compounds available for co-chromatography.

The assay used for individual carotenoid quantification has proved repeatable when analyzing plant material with a 4% error, whereas trans-β-carotene carried through the whole saponification-chromatographic procedure was recoverable at 92% or better with about 1.5% being converted to the difuranoid and other oxidation products. No isomerization occurred.

The possibility exists that a fecal "grab" sample is not representative of the total carotenoid content of the feces. Carotenoids are unstable in the presence of oxygen, however, and it was felt more important to assay a rectal sample than to mix all of the feces from a 1-day collection after having been exposed to air for various amounts of time.

It is generally accepted that β-carotene exists in plant material in a protein complex. To explore the possibility that this protein was somehow rendering the β-carotene unextractable in haylage, peptic digests, simulating the conditions found in the abomasum, were done on the 1966 haylage.

The initial digests were followed by individual analysis for each carotenoid. Since all the major carotenoids apparently increased with no outstanding decreases (table 2), the subsequent duplicate digests plus controls were run using only the AOAC (4) chromatographic technique following saponification and extraction to es-

TABLE 1

Carotenoid balance in heifers, 1 week collection, 1966

Animal no.	Diet	*Trans* β-carotene		Isomers β-carotene		Monoepoxide β-carotene		Monofuranoid β-carotene		Difuranoid β-carotene		Hydroxylated compounds [1]	
		Feed	Feces	Feed	Feces	Feed	Feces	Feed	Feces	Feed	Feces	Feed	Feces
		optical density units × 10³/week, measured at maximum absorption in hexane, 1 cm cuvette											
6	Haylage	78	290	8	160	13	18	0	6	23	21	300	570
8	Haylage	78	310	8	190	13	25	0	4	23	11	300	630
13	Haylage	75	120	8	71	12	11	0	4	22	12	290	600
36	Hay	210	330	76	114	36	12	13	22	11	2	690	680
41	Hay	120	130	43	47	21	8	8	6	6	4	390	330

[1] Compounds tentatively identified in decreasing concentrations: violaxanthin, lutein, isolutein, chrysanthemaxanthin, auroxanthin and neoxanthin.

timate β-carotene carotenoids (tables 3 and 4). Refluxing haylage samples with ethanol or ethanolic KOH before the regular extraction gave no increase of β-carotene and decreased total carotenoids.

To check the possibility that hay might also contain more β-carotene than initially extracted, a 16-hour peptic digest was done. No marked increase in the carotenoids occurred.

Feces from one animal fed haylage (no. 6) were also digested in vitro with pepsin since the apparent carotene content in the haylage incubated with acid increased as the amount of digestion time increased, even beyond the 4 to 6 hour duration that feed is in an acid environment in the abomasum and upper intestine. Following 16 hours of incubation in vitro carotenoids in the feces of cattle fed haylage increased 14 optical density (OD) units/g dry matter (DM); however, the initial fecal carotene value was 70 OD units and 1 g of fecal DM was equivalent to 2.4 g of haylage DM. Thus, this increase (14 OD units/g DM) in fecal carotenoids does not approach that found in the haylage samples (11 to 15 OD units/g DM) incubated under the same conditions.

DISCUSSION

Early work done by Whitnah et al. (5) with dairy cattle fed various rations showed a greater carotene excretion than intake, sometimes as high as 1,495%, although more often 240% or less. This was attributed to poor extraction of feed material or synthesis of a substance measured as carotene. More recently, McGillivray (6) reported fecal carotene content reaching 160% of the carotene found in the feed consumed by sheep. He suggested that this was due to carotene synthesis by the microorganisms present in the ileum and cecum.

The results reported here confirm an apparent increased fecal output of *trans*-β-carotene over the feed input with haylage, sometimes as high as 400%. The other carotenoids in general showed increases also. Even some of the acid-labile pigments such as the epoxides and furanoids of carotene followed this trend, although not to the extent or as consistently as the other carotenoids. Since hydroxy compounds

TABLE 2

Carotenoid content of haylage 1966, before and after peptic digest in vitro [1]

Carotenid	No digestion	Peptic digest, 16 hr, ether preextraction	Peptic digest, 16 hr, no preextraction
	optical density units per gram dry matter, measured at maximum absorption in hexane, 1 cm cuvette		
Neo β-carotene U	0.2	0.3	0.4
Trans β-carotene	2.4	4.2	2.5
Neo β-carotene B	0	0.5	1.0
β-Carotene monoepoxide	0.3	0	0
β-Carotene monofuranoid	0	1.1	0.4
β-Carotene diepoxide	+	0	0
β-Carotene difuranoid	0.7	0	0
Lutein, isolutein, chrysanthemaxanthin [2]	2.8	2.5	1.1
Violaxanthin	6.6	12.8	8.5
Auroxanthin	0	0.4	0.3

[1] One gram sample, 100 ml 0.2% pepsin, 0.075 N HCl, 40°, 16 hours, bubbling N_2 through sample.
[2] Quantification and identification of these compounds are only tentative as they were difficult to free from a substance absorbing in the ultraviolet region which grossly changed the absorption spectra of the carotenoids. Optical density measured at 440 mμ.

found in hay and haylage are present mostly as epoxides and furanoids, the acid conditions and resulting destructive environment in the abomasum may account for the smaller increase in their fecal output over input when compared with carotene.

Another observation made was the increase in the gut of the isomers of β-carotene. McGillivray (6) had previously reported no difference in the absorption spectrum between the carotene eaten and the carotene excreted in the feces. This implied that no isomerization occurred in the gut. With haylage, the isomers increased 10.5 to 24.0 times the amount fed. This may be explained by the acid conditions of the abomasum causing isomerization of the *trans*-β-carotene. A similar effect was demonstrated in the peptic digest in vitro with the increase being four to seven times for the isomers as compared with two or less for the *trans*-β-carotene.

Digestion of the haylage for 16 hours in vitro with hydrochloric acid at pH 2 doubled the measurable β-carotene. Pepsin did not appear to enhance this and the higher pH samples did not yield as great an increase as pH 2. Generally, the lower the pH of the incubation, the more carotene there was available for extraction; however, even the samples incubated at pH 9 had an apparently higher carotene content than the original material. Preextraction with ether or acetone–Skellysolve-B increased the total β-carotene measured both in the hydrochloric acid and water incubations but samples at higher pHs were only increased by one-half or less that of the pH 2 sample. This apparent increase of carotene by preextraction indicates that the carotene that is extractable before digestion may also be easily destroyed (and thus partially lost during incubation) since repeated pre- and post-extractions did not increase the yield of pigments. It may also be that preextraction removes destructive, lipid-soluble compounds that when not removed cause pigment destruction during subsequent incubation. Adding 65 IU α-tocopheryl acetate to the incubations, however, made no difference in the final values of any of the treatments. Temperature also had no marked effect on this loss of carotenoids.

Freeze-drying of the sample did not affect the amount of carotenoids that could be extracted, as frozen, fresh material assayed the same as freeze-dried samples.

The AOAC technique is not ideal for β-carotene estimation because it has been found in this laboratory that the "carotene" eluted from the magnesia column with 10% acetone in Skellysolve-B contains not only the isomers of β-carotene but also epoxides and furanoids of β-carotene (and α-carotene if present). These oxidation products may contribute as much as 15% of the total optical density measured at 450 mμ.[4] Because so little of the

[4] Unpublished data.

TABLE 3

β-Carotene content in haylage 1966, before and after incubation in vitro

Preextraction	0 hr	Pepsin,[1] 40°, pH 2		HCl,[2] 40°, pH 2		Water,[3] 40°, pH 5		Water,[3] 22°, pH 5		Water,[3] 4°, pH 5		Water,[4] 40°, pH 7	Water,[4] 40°, pH 9
		4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	16 hr	16 hr
		optical density units at 450 mμ/g dry matter in hexane, 1 cm cuvette [5]											
None		2.4	5.5	2.8	5.6	4.0 [7]	3.3	2.6	3.5	3.4	3.3	3.9	3.6
Ether [6]	3.0 ± 0.5	4.2	7.0	4.1 [7]	7.2 [7]	4.8	6.5	5.1	5.3	5.9	6.1	4.4	4.8
Acetone-Skellysolve-B [6]	2.9 ± 0.5	2.7	6.6	3.2	6.2	3.9	4.8	4.7	5.1	3.7	3.7		
Ether, acetone-Skellysolve-B [6]	3.6 ± 0.2	4.1	7.5										

[1] One gram sample, 100 ml 0.2% pepsin, 0.075 N HCl, bubbling CO_2 through.
[2] One gram sample, 100 ml 0.075 N HCl, bubbling CO_2 through.
[3] One gram sample, 100 ml distilled, deionized water with a normal pH of 5, CO_2 bubbling through.
[4] One gram sample, 100 ml 0.5 M Tris–HCl buffer, CO_2 bubbling through.
[5] Values are means of two or three samples with a range of less than 10% unless noted otherwise.
[6] Values include preextracted carotene.
[7] Range between 10 and 20%.

TABLE 4

Total carotenoid content in haylage 1966, before and after incubation in vitro

Preextraction	0 hr	Pepsin,[1] 40°, pH 2		HCl,[2] 40°, pH 2		Water,[3] 40°, pH 5		Water,[3] 22°, pH 5		Water,[3] 4°, pH 5		Water,[4] 40°, pH 7	Water,[4] 40°, pH 9
		4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	4 hr	16 hr	16 hr	16 hr
		optical density units at 450 mμ/g dry matter in hexane, 1 cm cuvette [5]											
None		18.5 [6]	28.6 [7]	18.5 [7]	27.1	25.9	16.6	12.6	30.5	18.2	16.7	12.6	15.8
Ether [8]	15.0 ± 1.5	25.2	36.8 [7]	25.1	31.6 [7]	26.3	31.8 [7]	35.6	36.2	41.6	43.0	16.0	20.1
Acetone-Skellysolve-B [8]	16.5 ± 4.0	16.7 [6]	30.5	24.0	25.7	22.5	28.2	27.9	30.0	18.7	20.8		
Ether, acetone-Skellysolve-B [8]	17.7 ± 1.5	20.8	35.2										

[1] One gram sample, 100 ml 0.2% pepsin, 0.075 N HCl, CO_2 bubbling through.
[2] One gram sample, 100 ml 0.075 N HCl, CO_2 bubbling through.
[3] One gram sample, 100 ml deionized water with a normal pH of 5, CO_2 bubbling through.
[4] One gram sample, 100 ml 0.5 M Tris–HCl buffer, CO_2 bubbling through.
[5] Values are means of two or three samples with a range of less than 10% unless noted otherwise.
[6] Range between 20 and 40%.
[7] Range between 10 and 20%.
[8] Values include preextracted carotene.

pigment in the digests was these compounds, however, it was felt that the AOAC technique was adequate for routine use in the digestion studies in vitro.

The apparent increase in carotenoid content of feces (from an animal fed haylage) after peptic digest indicates that not all of the carotene from the haylage has been made "available" by the animal. The lack of increase through in vitro incubation for the hay agreed with the in vivo results.

Because of the apparent parallel increases of the various individual pigments within both the carotene and hydroxylated fractions upon acid treatment, and the similarity between the proportions of the various pigments in the feed and feces, it is more likely that most if not all of the apparent synthesis reported by various workers is associated with inefficiencies in pigment extraction rather than microbial synthesis.

The carotene values obtained after in vitro incubation of the haylage approximate those found for the hay (7.0 OD units/g DM) with or without in vitro incubation. Even after correction for the in vitro results two of the animals fed haylage were apparently still excreting twice as much pigment as consumed, whereas the animals fed hay and one fed haylage were excreting an amount equal to their intake. Why this difference occurs has not yet been determined.

Presumably this additional carotene, plus much of that measurable in the haylage without acid treatment, is not available to the animals as evidenced by the decrease of their liver vitamin A levels during the studies. Nor does this binding of the carotenoids occur every year since the carotene content of the haylage made in 1967 was not increased by acid incubation nor was it excreted in greater quantities than ingested by sheep being fed the haylage. The β-carotene content of this haylage, however, was 20 OD units/g or about three times the maximum amount extractable from the 1966 haylage even after acid treatment. Whether this was due to the slightly higher percentage of grasses, a weather factor, a difference in field conditions, or the fact that a conventional silo was used has not been determinable.

LITERATURE CITED

1. National Research Council 1959 Nutrient requirements of domestic animals. 4. Nutrient requirements of beef cattle. National Academy of Science — National Research Council, Washington, D. C.
2. Curl, A. L., and G. E. Bailey 1961 Carotenoid epoxide detection: An improved test of epoxides. J. Agr. Food Chem., 9: 403.
3. Jungalwala, F. B., and H. R. Cama 1962 Carotenoids in *Delonix regia* (Gul Mohr) flower. Biochem. J., 85: 1.
4. Association of Official Agricultural Chemists 1965 Official Methods of Analysis, ed. 10. Washington, D. C.
5. Whitnah, C. H., W. J. Peterson, F. W. Atkeson and H. W. Cave 1939 Carotene balance and blood-carotene levels in heifers and lactating dairy cows, and their relation to the production of off-flavor milk. J. Agr. Res., 58: 343.
6. McGillivray, W. A. 1951 The apparent intestinal synthesis of carotene by sheep. Brit. J. Nutr., 5: 223.

Influence of Diet on Indirect Systolic Blood Pressure of Rats [1,2]

RICHARD L. ENGEN AND MELVIN J. SWENSON
Department of Physiology and Pharmacology, College of Veterinary Medicine, Iowa State University, Ames, Iowa

ABSTRACT This study was conducted to determine the influence of protein, vitamin B_{12} and choline on the systolic blood pressure of rats. Weanling rats were fed basal diets containing 10 or 25% protein and deficient in either vitamin B_{12} or choline. After 6 months on the basal diet, one-third of each dietary group was fed a diet containing 1% cholesterol, one-third was fed a diet containing 1% cholesterol plus 12% additional hydrogenated coconut oil and one-third continued on the assigned ration for the remaining 6 months of the experiment. Indirect systolic blood pressures were measured during months 9, 11 and 12 of the experiment. By averaging the blood pressure values of all three measurements, the male pressures were higher ($P < 0.01$) than the female, 130.8 and 124.1 mm Hg, respectively. The male blood pressures were greater for each determination than the female at 9, 11 and 12 months. The blood pressures and corresponding body weights for months 9 and 11 were correlated at the 1 and 5% level, respectively. All data were summarized as follows: males and females fed 25% protein, 131.3 mm Hg, and 10% protein, 123.6 mm Hg; males fed 25% protein, 136.7 mm Hg, and 10% protein, 125.0 mm Hg; and females fed 25% protein, 126.1 mm Hg, and 10% protein, 122.2 mm Hg. The 25% protein diet elevated the systolic pressure of the male rats. Cholesterol and hydrogenated coconut oil supplementation did not alter the indirect systolic blood pressure.

Dietary saturated fats, cholesterol and sucrose have been shown to cause hypercholesteremia and increase the incidence of atherosclerosis (1–4). The development of fibrous and calcified arteries during atherosclerosis decreases arterial distensibility causing hypertension. Research workers (5–9) have found that stenosis of the cerebral, carotid and vertebral arteries produces elevated systemic blood pressures. Age and obesity appear to be directly correlated to blood pressure in males (10–12), but females appear to be exempt until menopause or bilateral oophorectomy (9).

Low dietary protein, vitamin B complex deficiency and brewer's yeast supplementation have been shown to reduce the systemic blood pressure in abnormal physiological conditions (13–15),whereas dietary sodium chloride (16–19) and cadmium (20) have been demonstrated to produce hypertension. The biological responses obtained from specific diets for reducing experimentally produced hypertension are of questionable significance for the normal physiological condition. Therefore, in this study, the diets were evaluated for hyper- or hypotensive properties with the intact animal. The data indicate that long-term feeding of a diet can influence the systemic blood pressure.

MATERIALS AND METHODS

A total of 360 weanling rats of the Sprague-Dawley strain was placed in cages, two of the same sex to a cage. After the rats were separated into six groups (60 rats total with 30 males and 30 females), each group was fed one of the following diets for 6 months: 1) 10% protein; 2) 25% protein; 3) 10% protein-vitamin B_{12} deficient; 4) 25% protein-vitamin B_{12} deficient; 5) 10% protein-choline deficient; and 6) 25% protein-choline deficient.

After 6 months, each of the six groups was divided into three additional groups (20 rats, 10 males and 10 females) and treated as follows: 1) no supplementation, 2) 1% cholesterol and 3) 1% cholesterol plus 12% hydrogenated coconut oil. The animals were fed these supplemented diets for an additional 6 months.

Diet composition. The percentage of ingredients in the 10 and 25% protein diet is described in table 1.

Received for publication June 28, 1968.
[1] Supported by the Iowa Heart Association.
[2] Department paper no. 564.

TABLE 1
Diet composition of 10 and 25% protein diets

	10% Protein	25% Protein
	%	%
Casein (devitaminized)	11.78	29.40
Dextrose	70.02	52.40
Hydrogenated coconut oil	8.00	8.00
Vitamin mix [1]	0.10	0.10
Mineral mix [2]	5.00	5.00
Cellulose	5.00	5.00
Choline chloride [3]	0.10	0.10
	100.00	100.00

[1] Vitamin mix was prepared to meet NRC (21) requirements for the rat. Two mixes were prepared — one for the vitamin B_{12}-deficient diet and one for the vitamin B_{12}-adequate diet. Vitamin A palmitate (250,000 IU/g), 1.5%; vitamin D_2 (500,000 IU/g), 0.4%; thiamine·HCl, 0.5%; riboflavin, 0.8%; niacin, 4.0%; pyridoxine, 0.5%; Ca-pantothenate, 4.0%; biotin, 0.04%; folic acid, 0.2%; menadione, 0.5%; cyanocobalamin (B_{12}), 0.003%; inositol, 10.0%; p-aminobenzoic acid, 10.0%; cornstarch, 65.36%; and α-tocopherol succinate, 2.2%.
[2] Mineral mix from Jones, J. H., and C. Foster (22).
[3] Choline chloride was deleted in the choline-deficient diets.

Cellulose, a nondigestible filler, was increased and dextrose was decreased proportionally to achieve an equal caloric density in the diets containing the additional coconut oil. The diets were fed ad libitum.

Because a typical choline deficiency syndrome was manifested in the rats fed the low protein and choline-deficient diet and not in the high protein and choline-deficient group, the low protein group was supplemented with choline chloride for 2 weeks and then the level was gradually reduced to a final level of 0.25 mg/g diet. The syndrome was characterized by high mortality losses about 6 to 9 days after being placed on the low protein and choline-deficient diet. The losses were much higher in the male than in the female rats. The group losses were replaced with extra rats.

The data for this research project were analyzed statistically by an analyses of variance program with a computer.

Blood pressure determinations. The indirect tail blood pressures of rats were determined with a pneumatic pulse transducer.[3] Because the rat can control blood flow to the tail, it is necessary to employ methods for insuring caudal artery pulse. Research workers (23–25) have found that heating the ventral surface of the rat and tail to 40 or 41° enhances normal blood flow. The rats were placed in holding cages with a heating plate as a base. The cage

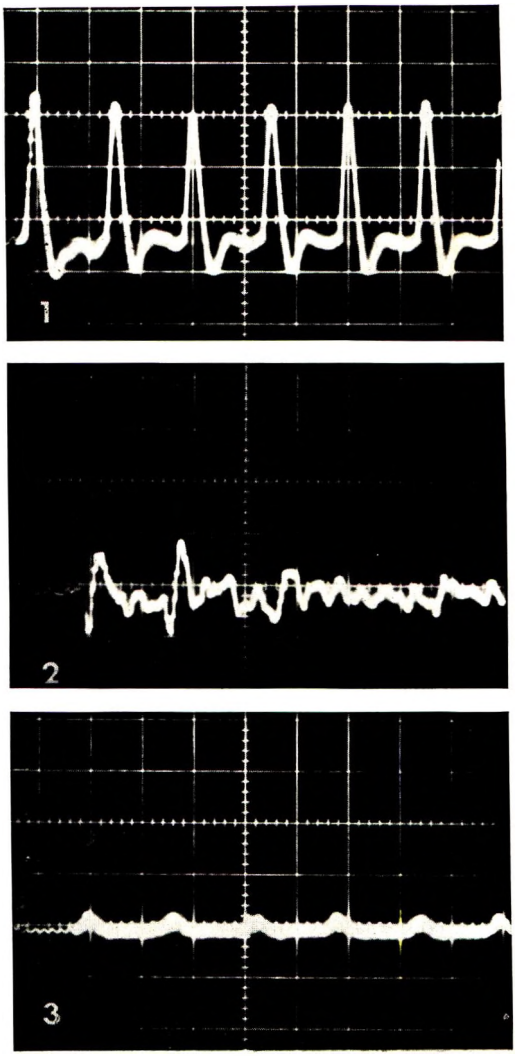


Fig. 1 The presence of the arterial pulse (1), the absence of the arterial pulse when the tail was occluded (2) and the initial reappearance of the arterial pulse after the tail cuff pressure was released (3).

was designed to ensure that the ventral surface of the rat and 3 to 6 cm of the tail would be on the heating plate. By using four holdings cages, cage adjustment time was sufficiently long (6 to 12 minutes) to reduce excitement and to record a sta-

[3] E and M Instrument Company, Houston, Tex.

bilized blood pressure value. A tail cuff attached to a pressure gauge was employed for occluding the tail arterial pulse. By increasing and decreasing the tail cuff pressure, the tail arterial pulse would disappear and reappear on the oscilloscope screen (fig. 1). The reappearance of the tail pulse was correlated to the pressure in the tail occluding cuff gauge and was recorded as indirect systolic pressure. Two readings were determined on each rat during months 9, 11 and 12 on test.

RESULTS AND DISCUSSION

The collated data (table 2) revealed that the male rat had a higher ($P < 0.01$) systolic blood pressure than the female rat. By combining the three determinations, the males averaged 130.8 and the females 124.1 mm Hg. Contrary to data from this project, Schroeder (20) demonstrated that female rats were more susceptible to hypertension caused by cadmium than male rats. After long duration cadmium feeding, the male rats gradually reached the state of hypertension exhibited earlier by the female rats. The conflicting data demonstrate more clearly the involvement of sex and diet with the systemic blood pressure. The results of this project indicate that the female rat on these diets has a resistance to the hypertension exhibited by the male rat. An explanation for these conflicting data is not apparent. The 25% protein diet increased ($P < 0.01$) the indirect systolic pressure of both sexes in month 9, and only the males in months 11 and 12.

Correlation coefficients were significant in both sexes between body weight (table 3) and blood pressure for month 9 ($P < 0.01$) and month 11 ($P < 0.05$). Hypertension, as it exists in this experiment, appears to be directly correlated with the body weight of the animal. Because dietary protein influences both body weight and blood characteristics, it was not possible to determine in this study if these hypertensive responses were directly related to body weight. The influence of sex on the cardiovascular system, however, was characterized by a lower systolic pressure in females than in males. Also, females maintained a lower systolic pressure than males fed the same 25% protein diet. The combination of high protein diet and the male sex elevated the systemic blood pressure.

The dietary supplementation of cholesterol and cholesterol plus additional coconut oil did not alter the indirect systolic blood pressure of the rat. A vitamin B_{12} deficiency and a choline deficiency caused an elevation in the blood pressure in the low protein-cholesterol supplemented group, but not in the cholesterol plus fat-supplemented group. It appears from these data that cholesterol supplementation alone promotes much greater stress than cholesterol plus fat. The elevated blood pressure occurred in both sexes at month 9 and in the males at the 11-month measurements. A possible explanation for this relationship may exist in the choline-low protein-cholesterol relationship. The combination of low protein and added cholesterol stressed the synthesis of choline in vivo for lipid transport. When a diminished synthesis of choline was combined with a dietary choline deficiency, the formation of the parasympathetic hormone, acetylcholine, was greatly reduced. A reduction in the concentration of acetylcholine would upset autonomic nervous system balance causing an elevation in the systemic blood pressure via sympathetic tone. This hypothesis is supported by Nagler et al. (26) who demonstrated a tissue decrease of acetylcholine in choline-deficient animals, and stated that the vascular system may be more susceptible to sympathetic tone. The reason for the elevation of blood pressure with vitamin B_{12} is not clear at this time.

In conclusion it appears that 1) the indirect systolic blood pressure was correlated with body weight in two of the three blood pressure determinations in both protein groups; 2) the dietary supplementation of protein elevates the systolic blood pressure of the male rats; and 3) neither cholesterol alone nor with additional coconut oil supplementation increased the indirect systolic blood pressure.

ACKNOWLEDGMENTS

The authors thank Dr. Donald Hotchkiss and Ralph Folsom for assistance in the computer programming for the statistical analyses, Corn Products of Illinois, Sheffield Chemical of New York, and Procter and Gamble of Ohio for supplying diet ingredients.

TABLE 2

Systolic blood pressure of rats

Sex	Month on test	Diets					
		10% protein	25% protein	10% protein-vitamin B_{12} deficient	25% protein-vitamin B_{12} deficient	10% protein-choline deficient	25% protein-choline deficient
		mm Hg	*mm Hg*	*mm Hg*	*mm Hg*	*mm Hg*	*mm Hg*
				Basal group			
Male	9	116.4 ± 3.2 [1]	134.5 ± 1.2	120.3 ± 4.2	136.1 ± 4.6	119.0 ± 8.0	140.5 ± 3.3
	11	126.3 ± 2.0	139.9 ± 6.7	130.9 ± 2.3	136.5 ± 4.7	132.9 ± 2.5	143.5 ± 4.0
	12	122.3 ± 2.2	137.3 ± 1.6	123.8 ± 2.7	133.4 ± 3.5	123.4 ± 4.7	133.9 ± 4.6
	avg	121.7 ± 2.5	137.2 ± 3.2	125.0 ± 3.1	135.3 ± 4.3	125.1 ± 5.1	139.3 ± 4.0
Female	9	119.5 ± 2.4	130.8 ± 5.3	114.8 ± 5.1	134.4 ± 2.3	108.9 ± 6.7	125.4 ± 4.9
	11	130.0 ± 2.1	128.4 ± 3.6	123.3 ± 3.0	127.7 ± 7.4	122.2 ± 4.0	134.8 ± 1.5
	12	131.9 ± 3.6	125.0 ± 2.0	121.4 ± 2.8	125.5 ± 2.8	122.5 ± 1.5	132.5 ± 1.8
	avg	127.1 ± 2.7	128.1 ± 3.6	119.8 ± 3.6	129.2 ± 4.2	117.9 ± 4.1	130.9 ± 2.7
				Cholesterol-supplemented group			
Male	9	119.0 ± 3.6	135.3 ± 4.6	121.7 ± 2.7	128.5 ± 1.5	131.9 ± 2.9	134.4 ± 2.2
	11	113.5 ± 3.9	137.9 ± 7.0	131.7 ± 2.5	136.8 ± 4.3	129.3 ± 3.6	139.3 ± 1.9
	12	118.4 ± 2.4	129.6 ± 4.1	127.0 ± 1.9	129.8 ± 3.3	129.0 ± 5.7	134.0 ± 3.4
	avg	117.0 ± 3.3	134.3 ± 5.2	126.8 ± 2.4	131.7 ± 3.0	130.1 ± 4.1	135.9 ± 2.5
Female	9	111.4 ± 3.5	125.5 ± 2.8	118.0 ± 3.8	126.2 ± 4.6	123.7 ± 5.5	126.8 ± 1.7
	11	119.8 ± 2.2	131.7 ± 4.5	127.4 ± 2.5	130.2 ± 7.1	126.7 ± 4.2	127.8 ± 1.3
	12	122.1 ± 2.6	119.4 ± 3.1	120.3 ± 4.6	122.5 ± 6.5	119.1 ± 3.7	123.8 ± 4.2
	avg	117.8 ± 2.8	125.5 ± 3.5	121.9 ± 3.6	126.3 ± 6.1	123.2 ± 4.5	126.1 ± 2.4
				Cholesterol + fat-supplemented group			
Male	9	130.5 ± 4.3	142.1 ± 1.3	129.3 ± 6.7	128.5 ± 4.1	120.5 ± 5.0	144.2 ± 4.1
	11	122.3 ± 1.8	139.9 ± 2.0	133.4 ± 8.5	143.1 ± 2.1	118.1 ± 2.2	136.5 ± 4.4
	12	124.7 ± 3.4	131.7 ± 2.1	133.2 ± 5.9	136.9 ± 4.0	128.2 ± 5.0	143.4 ± 3.7
	avg	125.8 ± 3.2	137.9 ± 1.8	132.0 ± 7.0	136.2 ± 3.4	122.3 ± 4.1	141.4 ± 4.1
Female	9	124.0 ± 3.5	127.1 ± 4.1	130.2 ± 4.0	126.5 ± 4.6	123.3 ± 2.9	126.8 ± 3.1
	11	121.4 ± 4.6	123.6 ± 3.3	121.8 ± 4.1	120.8 ± 3.2	118.4 ± 1.5	113.1 ± 4.7
	12	132.7 ± 4.1	123.4 ± 2.8	121.6 ± 3.7	123.7 ± 3.7	122.4 ± 3.0	121.3 ± 2.4
	avg	126.0 ± 4.1	124.7 ± 3.4	124.5 ± 3.9	123.7 ± 3.8	121.4 ± 2.5	120.4 ± 3.4

[1] ± = SE of the mean.

TABLE 3

Body weight of rats

Sex	Month on test	Diets					
		10% protein	25% protein	10% protein-vitamin B_{12} deficient	25% protein-vitamin B_{12} deficient	10% protein-choline deficient	25% protein-choline deficient
		g	*g*	*g*	*g*	*g*	*g*
				Basal group			
Male	9	244 ± 5 [1]	332 ± 14	251 ± 11	322 ± 11	211 ± 13	325 ± 17
	11	276 ± 5	398 ± 12	298 ± 10	360 ± 11	263 ± 8	365 ± 18
	12	280 ± 2	401 ± 11	299 ± 11	361 ± 8	266 ± 8	367 ± 16
	avg	267 ± 4	377 ± 12	283 ± 11	348 ± 10	247 ± 10	352 ± 17
Female	9	206 ± 9	235 ± 4	205 ± 6	237 ± 6	205 ± 2	245 ± 23
	11	222 ± 9	255 ± 5	222 ± 7	251 ± 6	214 ± 5	231 ± 6
	12	223 ± 8	251 ± 6	223 ± 6	250 ± 5	214 ± 6	234 ± 3
	avg	217 ± 9	247 ± 5	217 ± 6	246 ± 6	211 ± 4	237 ± 11
				Cholesterol-supplemented group			
Male	9	222 ± 13	299 ± 15	234 ± 10	291 ± 9	184 ± 5	281 ± 16
	11	257 ± 16	310 ± 25	272 ± 6	331 ± 9	246 ± 8	312 ± 14
	12	252 ± 16	335 ± 18	278 ± 5	336 ± 7	254 ± 5	312 ± 10
	avg	244 ± 15	315 ± 19	261 ± 7	319 ± 8	228 ± 6	302 ± 13
Female	9	185 ± 9	235 ± 11	201 ± 9	230 ± 5	149 ± 7	210 ± 6
	11	206 ± 3	256 ± 6	217 ± 7	237 ± 4	179 ± 5	222 ± 5
	12	207 ± 2	247 ± 8	213 ± 6	230 ± 6	188 ± 5	223 ± 7
	avg	199 ± 5	246 ± 8	210 ± 7	232 ± 5	172 ± 6	218 ± 6
				Cholesterol + fat-supplemented group			
Male	9	236 ± 8	319 ± 18	235 ± 9	317 ± 18	181 ± 10	296 ± 9
	11	267 ± 8	365 ± 10	265 ± 9	326 ± 12	200 ± 10	327 ± 6
	12	278 ± 8	376 ± 9	273 ± 11	333 ± 12	212 ± 13	336 ± 7
	avg	260 ± 8	353 ± 12	258 ± 10	325 ± 14	198 ± 11	320 ± 7
Female	9	197 ± 6	223 ± 10	186 ± 9	230 ± 5	165 ± 9	212 ± 8
	11	212 ± 6	232 ± 10	210 ± 12	243 ± 4	185 ± 6	221 ± 5
	12	216 ± 7	233 ± 7	220 ± 9	243 ± 4	196 ± 5	223 ± 7
	avg	208 ± 6	229 ± 9	205 ± 10	239 ± 4	182 ± 7	219 ± 7

[1] ± = SE of the mean.

LITERATURE CITED

1. Freedman, M., and S. O. Beyers 1963 Observation concerning the evaluation of atherosclerosis in the rabbit after cessation of cholesterol feeding. Amer. J. Pathol., *43:* 349.
2. Hill, E. G., E. L. Warmanen, H. Hayes and R. T. Holman 1957 Effects of essential fatty acid deficiency in young swine. Proc. Soc. Exp. Biol. Med., *95:* 274.
3. Lambert, G. E., J. P. Miller, R. T. Olsen and D. V. Frost 1958 Hypercholesteremia and atherosclerosis induced by purified high fat rations devoid of cholesterol. Proc. Soc. Exp. Biol. Med., *97:* 544.
4. Yudkin, J. 1957 Diet and coronary thrombosis — hypothesis and fact. Lancet, *2:* 155.
5. Dickinson, C. J., and A. D. Thomson 1960 A post-mortem study of the main cerebral arteries with special reference to their possible role in blood pressure regulation. Clin. Sci., *19:* 513.
6. Davis, D., and M. J. Klainer 1940 Studies in hypertensive heart disease. I. The incidence of coronary atherosclerosis in cases of essential hypertension. 2. The role of hypertension per se in the development of coronary stenosis. Amer. Heart J., *19:* 185.
7. Evans, P. H. 1965 Relation to long standing blood pressure levels to atherosclerosis. Lancet, *1:* 516.
8. Wilkins, R. H., J. C. Roberts and C. Moses 1959 Autopsy studies in atherosclerosis. III. Distribution and severity of atherosclerosis in the presence of obesity, hypertension, nephrosclerosis, and rheumatic heart disease. Circulation, *20:* 527.
9. Mitchell, J. R. A., C. J. Schwartz and A. Zinger 1964 Relationship between aortic plaques and age, sex, and blood pressure. Brit. Med. J., *1:* 205.
10. Brown, M. L., M. Roostein and F. D. Zeman 1963 The relationship of changes of blood and pulse pressure to cardiovascular status in 100 aged individuals. Amer. J. Med. Sci., *245:* 676.
11. Keys, A., H. L. Taylor, H. Blackburn, J. Brozek, J. T. Anderson and E. Simonson 1963 Coronary heart disease among Minnesota business and professional men followed fifteen years. Circulation, *28:* 381.
12. Master, A. M., C. I. Garfield and M. B. Walters 1952 Normal Blood Pressure and Hypertension. Lea and Febiger, Philadelphia.
13. Calder, R. M. 1942 Nutritional deficiencies as a cause of elevated blood pressure in rats (with special reference to the vitamin B_{12} complex). J. Nutr., *76:* 1.
14. Handler, P., and F. Bernheim 1950 Physiological basis for effects of low-protein diets on blood pressure of subtotally nephrectomized rat. Amer. J. Physiol., *162:* 368.
15. DeKleine, W. 1944 Suggested inquiry into possible relation between albuminuria, essential hypertension, and nutritional deficiency. Mich. State Med. Soc. J., *44:* 897.
16. Koletsky, S. 1958 Hypertensive vascular disease produced by salt. Lab. Invest., *7:* 377.
17. Meneely, G. R., R. G. Tucker, W. J. Darby and S. H. Auerbach 1953 Chronic sodium chloride toxicity in the albino rat. II. Occurrence of hypertension and of a syndrome of edema and renal failure. J. Exp. Med., *98:* 71.
18. Sapirstein, L. A., W. L. Brandt and D. R. Durry 1950 Production of hypertension in the rat by substituting hypertonic sodium chloride solution for drinking water. Proc. Soc. Exp. Biol. Med., *73:* 82.
19. Hall, C. E., and O. Hall 1965 Hypertension and hypersalimentation. Lab. Invest., *14:* 285.
20. Schroeder, H. A. 1964 Cadmium hypertension in rats. Amer. J. Physiol., *207:* 62.
21. National Research Council 1962 Nutrient Requirements of Laboratory Animals, no. 10. National Academy of Sciences — National Research Council, Washington, D.C.
22. Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets low or high in phosphorus. J. Nutr., *24:* 245.
23. Olmsted, F. A., A. C. Corcoran and I. H. Page 1951 Blood pressure in the unanesthetized rat. II. Spontaneous variation and effect of heat. Circulation, *3:* 722.
24. Proskauer, A. A., C. Neuman and L. Graff 1945 Measurement of blood pressure in the rat with special reference to effect of changes in temperature. Amer. J. Physiol., *143:* 290.
25. Sobin, S. S. 1945 Accuracy of indirect determination of blood pressure in rats: relation of temperature of plethysmograph and width of cuff. Amer. J. Physiol., *146:* 179.
26. Nagler, A. L., W.-D. Dettbarn, E. Seifter and S. A. Levenson 1968 Tissue levels of acetylcholine and acetylcholinesterase in weanling rats subjected to acute choline deficiency. J. Nutr., *94:* 13.

Zinc-65 Uptake by Rumen Tissue

S. P. ARORA,[1] E. E. HATFIELD, U. S. GARRIGUS, T. G. LOHMAN AND B. B. DOANE

Animal Science Department, University of Illinois, Urbana, Illinois

ABSTRACT Studies in vivo and in vitro were conducted to determine the uptake of zinc by the rumen tissues of lamb. Absorption of ^{65}Zn by the rumen tissue was estimated following oral and intravenous administrations. The maximum uptake of ^{65}Zn by rumen tissue of orally administered and intravenously administered zinc was at 48 and 12 hours, respectively. The uptake of ^{65}Zn per kilogram of rumen tissue was significantly greater ($P < 0.01$) than the uptake per kilogram of abomasal, duodenal or intestinal tissue. One experiment in vitro (replicated twice) was carried out to determine ^{65}Zn uptake by rumen tissue. The mucosal surface of rumen tissue absorbed 71.2 ± 2.9% ^{65}Zn from buffered nutrient solutions. The results indicated that rumen tissue zinc uptake might be a process of absorption other than the concentration gradient.

Zinc has been associated with protein in several biological systems, particularly enzymes. Several zinc metalloenzymes have been characterized (1), but information regarding the transport mechanisms of this mineral in mammals after ingestion is still incomplete. It has been demonstrated that ^{65}Zn was secreted into the rumen contents of lambs after intravenous dosage (2). Orally administered zinc appears to be absorbed at different rates by various parts of the digestive tract in dairy cattle (3). More evidence is needed before concluding that some organs of the gastrointestinal tract absorb and retain zinc to a greater extent than other organs.

These studies in vivo and in vitro were designed to determine the uptake of zinc by the rumen tissue of lambs.

EXPERIMENTAL PROCEDURE

Experiment 1. Twelve Rambouillet × Hampshire lambs, 9 months old and weighing 29 kg, were fed 1 kg of a low zinc diet (table 1) per head per day for 44 days to prevent perturbance of administered ^{65}Zn by homeostatic controls because zinc salt had been shown to accelerate the excretion of ^{65}Zn through the gastrointestinal tract (4). The zinc content of the diet was approximately 23 ppm. All precautions were taken as far as possible to prevent the lambs from gaining access to any material containing zinc. A single oral dose of 200 μCi of ^{65}Zn and 48 mg of stable zinc (as $ZnCl_2$) in gelatin capsule was given to each lamb. The lambs were fed the same diet at the time ^{65}Zn was given.

The ^{65}Zn was in the compound $^{65}ZnCl_2$ and had a specific activity of 2.49 mCi/mg of zinc which was diluted with distilled water to contain 400 μCi/ml. A 200-μCi reference standard was taken to compare with tissue samples collected at timed intervals. Negative control animals were not used as ^{65}Zn was not detected in tissue of animals not administered ^{65}Zn (5). Two randomly selected lambs were killed at each time interval after the administration of ^{65}Zn. The animals were killed by severing the jugular vein after they had been anesthetized by an intravenous injection of sodium pentobarbital. Rumen tissue samples were taken from these lambs and thoroughly washed with running tap water. Blood was also collected at the time of killing. The timed intervals were 12, 24, 48, 96, 144 and 192 hours after ^{65}Zn administration. Radioactivity of the tissue samples and radioactivity of the reference standard were measured with a scintillation counter [2] (6). The radioactivity of the tissue was expressed as a percentage of the radioactivity of the reference standard and afterwards calculated per kilogram tissue. All tissue samples and the reference standard were monitored for radioactivity in

Received for publication June 15, 1968.

[1] Indian staff member from J. L. N. Agriculture University, Jabalpur, M. P., assigned to study at the University of Illinois in Animal Science (Nutrition) under the A. I. D. program.

[2] Metrix, Inc., designed this apparatus for the University of Illinois, Urbana.

TABLE 1

Basal diet

Ingredient	Quantity
	% [1]
Corn, ground	52.1
Cellulose [2]	24.8
Soybean meal	19.9
Zinc-free mineral mixture [3]	3.0
Aurofac [4]	0.1
Quadrex [5]	0.1

[1] Calculated analysis: (in percent) protein, 15.0; and crude fiber, 31.9.
[2] Solka Floc (BW-40), Brown Company, Chicago, Illinois.
[3] Provided as percentage of the total diet: $CaHPO_4 \cdot 2H_2O$, 1.0481; KSO_4, 0.89; NaCl, 0.5; Na_2SO_4, 0.35; MgO, 0.14; $FeSO_4 \cdot 7H_2O$, 0.06; MnO_2, 0.006; $CuSO_4 \cdot 5H_2O$, 0.0047; KI, 0.0011; and $CoSO_4 \cdot 7H_2O$, 0.0001.
[4] Contributed 22 mg chlortetracycline/kg of diet.
[5] Contributed per kilogram of diet: vitamin A, 10,000 IU; and vitamin D, 1,250 IU.

containers placed 1 cm from the NaI crystal.

Experiment 2. A single dose of 200 μCi ^{65}Zn (0.5 ml) and 48 mg of stable zinc was injected into the jugular vein to each of three lambs weighing 30 kg. The ^{65}Zn was in the form of $ZnCl_2$ in 3 N HCl (0.38 ml), diluted to 12.5 ml. The lambs were fed 1 kg of the low zinc diet per lamb per day for 39 days prior to the treatment and same rate of diet was continued during the treatment. At 3, 12 and 24 hours after zinc administration, a lamb was killed and tissue samples taken. Experimental procedure is the same as described in experiment 1.

Experiment 3. Because of highest concentration of ^{65}Zn in the rumen tissue at the 48-hour interval in experiment 1, another group of five lambs was killed 48 hours after oral administration of ^{65}Zn (200 μCi) and 48 mg stable zinc. The lambs weighed 35 kg and were fed a low zinc diet for 32 days at the rate of 1.2 kg/lamb per day prior to the treatment and the same rate of diet was continued during the treatment. As in experiment 1 tissue samples taken from the rumen, abomasum, duodenum and large intestine were used to measure relative radioactivity uptake.

Experiment 4. Studies in vitro were conducted with rumen sacs. Three rumen sacs were made from tissues taken from the ventral side of the rumen wall of three different lambs that had been receiving the low zinc diet. The sacs were made with the papillae side to the inside and the serosal surface to the outside. The free edges were sutured and sealed to provide anaerobic environment inside the sac. The three sacs were submerged in a physiological buffered medium [3] in 600-ml glass beakers. The buffered medium was also used inside the sac. Approximately 2 μCi of ^{65}Zn/100 g tissue were placed in the lumen of the sac with a tuberculin syringe. The rumen sac preparations were then incubated at 40° in a water bath for 4.75 hours. The sacs were carefully removed and the outer solutions and the inner solutions were placed in separate containers. The rumen tissue was thoroughly washed with running distilled water for 10 minutes and placed in a separate container. A reference standard of 2 μCi of ^{65}Zn was used to measure relative amounts of radioactivity in the tissue and buffered medium solution. The radioactivity of the rumen tissue, outer solution and inner solution, was determined and expressed as a percentage of the reference standard.

RESULTS AND DISCUSSION

At the time periods studied, the highest level of radioactivity of ^{65}Zn per kilogram of rumen tissue was at the 48-hour interval after oral administration. The decrease in the radioactivity after 48 hours appeared to be a function of time (fig. 1). The concentration of ^{65}Zn in the rumen tissue was greater than the concentration of ^{65}Zn in the blood. It is postulated that ^{65}Zn might be transferred from the rumen contents to the rumen wall tissue by the process of absorption. In experiment 2 with intravenous injections, the highest radioactivity of ^{65}Zn in the rumen tissue was at the 12- and 24-hour interval at the time periods studied. Higher concentrations of ^{65}Zn in the rumen were obtained by injection compared with oral dosing. As in experiment 1 the rumen tissue concentration of radioactivity was greater than the radioactivity of the blood (fig. 2). In experiment 3 the radioactivity per kilogram of tissue of the rumen tissue was significantly ($P < 0.01$) greater than abomasal, duodenal or large intestinal tissues (table 2). In the experiment in vitro with the rumen sacs, the mean radioactivity of the rumen tissue, expressed as a percentage of the reference

[3] Hanks' BSS buffer-medium, Grand Island Biological Company, Grand Island, New York.

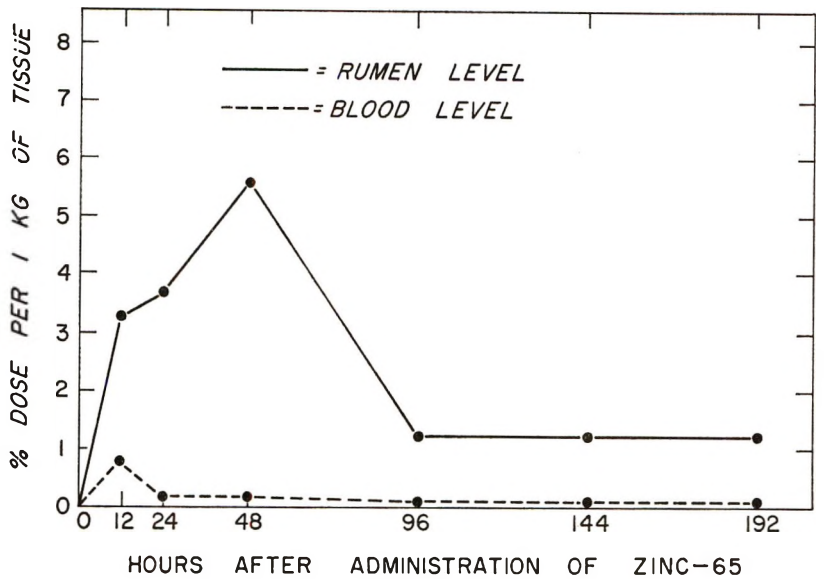


Fig. 1 Mean ^{65}Zn uptake per kilogram tissue of two lambs at each timed interval after oral administration.

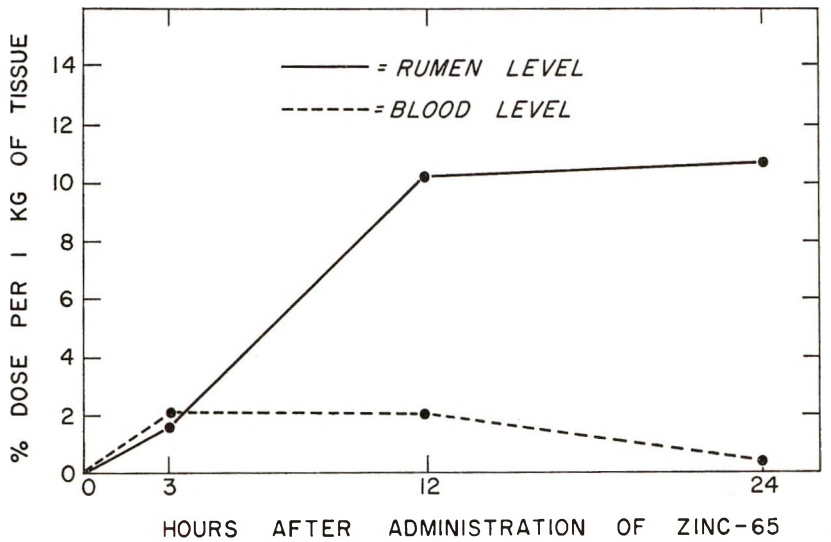


Fig. 2 The ^{65}Zn uptake per kilogram tissue of one lamb at each timed interval after intravenous injection.

standard was 71.2 ± 2.9% indicating that the ^{65}Zn was absorbed from the inner solution. Using the same technique to estimate the amount of radioactivity, it was shown that 22.9 ± 4.6% of the original dose was still in the inner solution. Measurements of the solutions on the outside of the rumen sacs indicated that only negligible amounts (0.15 ± 0.02%) were present by transfer through the rumen wall. These observations indicate that rumen tissue has the ability to absorb an appreciable amount of zinc in addition to the other sites of absorption such as abomasum and lower small intestine reported in dairy cattle (3). The experimental diet given to lambs in the

TABLE 2

Comparison of ^{65}Zn uptake in vivo in different parts of the alimentary tract

Tissue	Mean ^{65}Zn uptake per 1 kg [1]	Analysis of variance				
		Source of variation	df	SS	MSS	F
Rumen	4.56	TSS	19	69.55	3.5	
Abomasum	1.14	Treatment SS	3	35.52	11.84	5.56 [2]
Duodenum	1.64	Error SS	16	34.03	2.13	
Large intestine	1.82	Rumen × others	1	34.16	34.16	16.04 [2]
		Abomasum × duodenum and intestine	1	1.18	1.18	0.51
		Duodenum × intestine	1	0.14	0.14	0.07

[1] On wet basis without drying.
[2] Significant at 1% level.

present study contained a normal level of calcium for minimum effect on the mode of zinc absorption. This diet, however, might contain phytic acid because of its source from plants. Dietary phytic acid and calcium could exert a deleterious effect on the absorption of zinc, a condition reported in the growing chick (7–9), the pig (10) and the rat (11, 12). This might be due to the formation of an insoluble complex containing zinc, phytate and calcium (13). All these reports concerned monogastric animals, with one exception, where phytic acid had been shown not to affect the availability of zinc in lambs on a purified diet (14). Furthermore, dietary phytase and rumen microbiological activity could hydrolyze phytic acid in the alimentary tract, primarily in the rumen of sheep (15). Thus, there was only a slight chance of interference in the absorption of zinc by either calcium or phytic acid in this study. Therefore, zinc absorption in the light of present knowledge occurred with minimum involvement of any other factor.

LITERATURE CITED

1. Vallee, B. L. 1959 Biochemistry, physiology and pathology of zinc. Physiol. Rev., *39:* 443.
2. Weston, R. H., and J. Kastelic 1967 The transfer of zinc from the blood to the rumen in sheep. Australian J. Biol. Sci., *20:* 975.
3. Miller, J. K., and R. G. Cragle 1965 Gastrointestinal sites of absorption and endogenous secretion of zinc in dairy cattle. J. Dairy Sci., *48:* 370.
4. Cotzias, G. C., and P. S. Papavasliou 1964 Specificity of zinc pathway through the body: Homeostatic considerations. Amer. J. Physiol., *202:* 359.
5. Miller, W. J., D. M. Blackmon, R. P. Gentry, W. J. Pitts and G. W. Powell 1967 Absorption, excretion, and retention of orally administered zinc-65 in various tissues of zinc-deficient and normal goats and calves. J. Nutr., *92:* 71.
6. Twardock, A. R., T. G. Lohman, G. S. Smith and B. C. Breidenstein 1966 Symposium on atomic energy in animal science. I. The Illinois Animal Science Counter: Performance characteristics and animal radioactivity measurement procedures. J. Anim. Sci., *25:* 1209.
7. O'Dell, B. L., and J. E. Savage 1960 Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med., *103:* 304.
8. Maddiah, V. T., A. A. Kurnick and B. L. Reid 1964 Phytic acid studies. Proc. Soc. Exp. Biol. Med., *115:* 391.
9. Likuski, H. J. A., and R. M. Forbes 1964 Effect of phytic acid on the availability of zinc in amino acid and casein diets fed to chicks. J. Nutr., *84:* 145.
10. Oberleas, D., M. E. Muhrer and B. L. O'Dell 1962 Effects of phytic acid on zinc availability and parakeratosis in swine. J. Anim. Sci., *21:* 57.
11. Likuski, H. J. A., and R. M. Forbes 1965 Mineral utilization in the rat. IV. Effect of calcium and phytic acid on the utilization of dietary zinc. J. Nutr., *85:* 230.
12. Heth, D. A., and W. G. Hoekstra 1965 Zinc-65 absorption and turnover in rats. I. A procedure to determine zinc-65 absorption and antagonistic effect of calcium in a practical diet. J. Nutr., *85:* 367.
13. Ott, E. A., W. H. Smith, M. Stob and W. M. Beeson 1964 Zinc deficiency syndrome in the young lamb. J. Nutr., *82:* 41.
14. Oberleas, D., M. E. Muhrer and B. L. O'Dell 1966 Dietary metal-complexing agents and zinc availability in the rat. J. Nutr., *90:* 56.
15. Reid, R. L., M. C. Franklin and E. G. Hallsworth 1947 The utilization of phytate phosphorus by sheep. Australian Vet. J., *23:* 136.

Effect of Different Patterns of Excess Amino Acids on Performance of Chicks Fed Amino Acid-deficient Diets

M. SUGAHARA, D. H. BAKER AND H. M. SCOTT
Department of Animal Science, University of Illinois, Urbana, Illinois

ABSTRACT Two assays were conducted to determine the effects on chick growth of individual amino acid deficiencies. Deficiencies of phenylalanine-tyrosine, tryptophan or isoleucine impaired growth more than equal deficiencies of other amino acids. In assay 2 individual amino acids were held at 60% of the chick requirement and were compared with a total deficiency of amino acids wherein all amino acids were reduced to 60% of the chick requirement. Compared with total amino acid deficiency, weight gain was a) increased by individual deficiencies of methionine-cystine, leucine, lysine or arginine, b) not affected by a deficiency of histidine and c) decreased by deficiencies of phenylalanine-tyrosine, tryptophan, isoleucine, valine or threonine. Utilization of the deficient amino acid was greater when that amino acid alone was deficient than when all of the other amino acids were reduced to a comparable level of deficiency. Compared with the complete amino acid diets, food consumption was reduced when individual amino acids were made deficient but not when all amino acids were made deficient simultaneously. The pattern of amino acids in excess of that needed for growth appeared to dictate whether an amino acid imbalance would be superimposed on that of an amino acid deficiency.

It is sometimes assumed that dietary amino acids are equally limiting when their respective dietary concentrations bear the same relationship to the animal requirement. A considerable body of evidence has accumulated in this laboratory to indicate that chick diets formulated to be equally limiting in this manner are not necessarily equally limiting in terms of ability to promote growth. It has been observed that chicks gain faster when fed a diet deficient in leucine or arginine than when fed a diet equally deficient in isoleucine (1). Other work [1] has demonstrated that a diet designed specifically to be equally limiting in arginine, isoleucine and valine becomes first limiting in valine when imbalanced with excess leucine, first limiting in arginine when imbalanced with excess lysine and equally limiting in all three amino acids when threonine is the imbalancing amino acid.

It is readily apparent that strict adherence to a straightforward "chemical score" concept of amino acid limitation offers no explanation for many of the observations on amino acid deficiency or imbalance that have been reported. A deficiency of one amino acid may exert different effects on food intake, hence growth, than a deficiency of another amino acid calculated to be equally deficient, simply because the pattern of excess amino acids is different in each instance. Also bearing on this question is the relative proportion of a limiting amino acid used for maintenance or for growth. Thus, a deficiency of sulfur-containing amino acids for the chick might be expected to impair growth more than an equal deficiency of lysine, because the maintenance requirement of sulfur-containing amino acids is greater (relative to the need for growth) than the requirement of lysine.

The objective of the studies reported here was to evaluate the relative growth depression induced by feeding diets equally deficient in each of the classical 10 essential amino acids. The crystalline amino acid standard of Dean and Scott (2) and a reference standard developed from this were used as requirement reference points.

EXPERIMENTAL PROCEDURE

Pretreatment of chicks and assay procedures were the same as previously reported (2). A corn–soybean meal ration was fed during the first 7 days posthatch-

Received for publication July 29, 1968.

[1] Mathieu, D., and H. M. Scott 1968 Growth depressing effect of excess leucine in relation to the amino acid composition of the diet. Poultry Sci. (in press).

ing. Growth assays were initiated on day 8 and terminated on day 14. Other than crystalline amino acids (table 1) the experimental diets contained 15.00% corn oil, 3.00% cellulose, 5.37% salt mixture (2), 1.00% $NaHCO_3$, 0.20% choline chloride, 0.20% vitamin mix (2), 20 mg/kg α-tocopherol acetate, 125 mg/kg ethoxyquin, and corn starch to bring all diets to 100%.

Assay 1. Based upon individual assays, a reference standard amino acid pattern (RS) was developed from Dean's standard no. 3 (DS-3). Except for cystine, the concentration of each amino acid in RS is less than the level present in DS-3 (table 1). Repeated assays have demonstrated that growth of chicks fed DS-3 is about 1 to 2 g/day greater than that obtained when RS is fed. Feed consumption is similar when either standard is fed.

The assay was designed to compare chick performance when each of the essential amino acids was reduced from amino acid mixture DS-3 down to the level shown in amino acid mixture RS. These treatments were in turn compared with the weight gain noted when DS-3 and RS were fed.

Assay 2. In this assay, diets were formulated to contain a complete mixture of crystalline amino acids representing RS, a mixture containing 60% of RS (60% RS), or 60% RS supplemented with all amino acids (up to RS), except for the deficient amino acid which remained at 60% of the chick requirement. Differing slightly from assay 1, methionine and cystine were both reduced, as were phenylalanine and tyrosine, to 60% of the chick requirement.

RESULTS

The effect of reducing individual amino acids from the concentration in DS-3 down to the level in RS is shown, in order of increasing effect, in table 2. Reducing phenylalanine, isoleucine, or tryptophan down to the level of RS impaired gain more than that observed when all amino acids were reduced simultaneously. Single reductions of the other essential amino acids resulted in weight gains greater than those noted for RS but less than those for DS-3. When the level of a single essential amino acid was lowered, feed intake was less but feed conversion greater than that noted when the level of all essential amino acids was lowered.

Assay 2. Striking differences were observed in the performance of chicks fed diets with individual amino acids held at 60% of the chick requirement (table 3). In agreement with the results of assay 1, a deficiency of phenylalanine-tyrosine, isoleucine or tryptophan resulted in a marked growth depression when compared with a

TABLE 1

Composition of crystalline amino acid mixtures (% of diet)

Amino acid	DS-3 [1]	RS [2]	60% RS [3]
	%	%	%
L-Arginine·HCl	1.330	1.210	0.726
L-Histidine·HCl·H_2O	0.620	0.410	0.246
L-Lysine·HCl	1.400	1.190	0.714
L-Tyrosine	0.630	0.450	0.270
L-Tryptophan	0.225	0.150	0.090
L-Phenylalanine	0.680	0.500	0.300
DL-Methionine	0.550	0.350	0.210
L-Cystine	0.350	0.350	0.210
L-Threonine	0.850	0.650	0.390
L-Leucine	2.010	1.200	0.720
L-Isoleucine	0.900	0.600	0.360
L-Valine	1.040	0.820	0.492
Glycine	1.600	1.200	0.720
L-Proline	1.000	0.200	0.120
L-Glutamic acid	12.000	10.000	6.000
Total	25.185	19.280	11.568

[1] Dean's standard no. 3 (2).
[2] Reference standard.
[3] All amino acids reduced to 60% of level in reference standard.

TABLE 2

Weight gain, feed consumption and feed efficiency of chicks fed diets containing different amino acid patterns (assay 1) [1]

Amino acid reduced from DS-3 [2]	Gain	Feed consumption	Gain/Feed
	g/chick/day	*g/chick*	
All ≈ RS	12.53±0.13 [3]	115.7	0.65
Threonine	13.85±0.28	113.8	0.73
Leucine	13.47±0.53	107.8	0.75
Methionine	13.37±0.31	109.9	0.73
Histidine	13.32±0.19	111.0	0.72
Lysine	13.17±0.31	108.2	0.73
Arginine	13.15±0.33	109.6	0.72
Valine	12.75±0.39	107.7	0.71
Tryptophan	12.13±0.45	102.5	0.71
Isoleucine	11.20±0.50	101.8	0.66
Phenylalanine	10.75±0.23	93.5	0.69
None ≈ DS-3	14.13±0.08	111.6	0.76

[1] Three replicates of 10 male chicks/treatment.
[2] Amino acid levels were reduced by an amount equal to the difference between DS-3 and RS.
[3] Mean ± SE of mean.

TABLE 3

Weight gain, feed consumption and feed efficiency of chicks fed diets equally deficient in a single amino acid (assay 2) [1]

Amino acid held at 60% of RS	Gain	Feed consumption	Gain/Feed
	g/chick/day	*g/chick*	
All ≈ 60% RS	8.60±0.56 [2]	112.2	0.46
Methionine + cystine	9.89±0.46	104.1	0.57
Leucine	9.72±0.67	94.1	0.62
Lysine	9.54±0.51	102.2	0.56
Arginine	9.27±0.19	104.9	0.53
Histidine	8.24±0.41	91.6	0.54
Threonine	7.89±0.44	96.6	0.49
Valine	7.41±0.28	88.9	0.50
Isoleucine	6.12±0.55	81.6	0.45
Tryptophan	5.94±0.16	77.5	0.46
Phenylalanine + tyrosine	5.73±0.44	74.7	0.46
None ≈ RS	13.16±0.23	112.8	0.70

[1] Two replicates of 10 male chicks/treatment.
[2] Mean ± SE of mean.

deficiency of all amino acids as represented by 60% RS. Seemingly, another category of growth response was represented by valine and threonine whose deficiency effected a moderate growth depression compared with 60% RS. With the exception of histidine, a deficiency of which resulted in gains similar to 60% RS, chicks fed diets deficient in arginine, lysine, leucine or sulfur-bearing amino acids gained faster than those fed 60% RS. As in assay 1, single deficiencies decreased feed consumption but increased gain-to-feed ratios as compared with a total deficiency of amino acids. Chicks fed 60% RS consumed as much feed as those fed RS.

DISCUSSION

That a methionine deficiency or a methionine plus cystine deficiency resulted in a performance superior to deficiencies of most other amino acids (table 3) tends to negate the view often expressed that a more severe growth depression should occur from a deficiency of an amino acid whose maintenance requirement is high relative to its growth requirement. It is conceivable, however, that the high maintenance requirement for sulfur-containing amino acids reported for the adult male by Leveille et al. (3) may not apply to the young chick. Nevertheless, the overriding influence of the pattern of excess amino

acids appears to be a more plausible explanation for the varied response to a single amino acid deficiency. Moreover, based upon these data there seems to be no clear-cut explanation of why certain patterns of excess amino acids are more noxious than others.

It cannot be said that a single amino acid deficiency causes a greater growth depression than a deficiency of all amino acids. Indeed, some of the individual amino acid deficiencies improved gain compared with a total amino acid deficiency. Likewise, it cannot be concluded that absence of one of the more toxic amino acids, lysine, from the imbalancing mixture prevented a growth depression, since in both the leucine- and sulfur amino acid-deficient diets lysine was present in the imbalancing mixture, yet growth was not reduced as compared with the total amino acid deficiency. The data suggest that a pattern of excess amino acids devoid of either phenylalanine, isoleucine or tryptophan is more detrimental than a pattern resulting from omissions of other amino acids from a supplemental mixture. It follows also that supplementing a low nitrogen diet (60% RS) with a mixture of amino acids, devoid of the indispensable amino acid that is to become first limiting, does not necessarily imbalance the diet. As noted previously, in some instances growth rates were actually improved by omitting certain amino acids from the "imbalancing" mixture.

In most cases utilization of the deficient amino acid was greater when that amino acid alone was deficient than when all the other amino acids were reduced to a comparable level of deficiency. This was particularly true in the case of methionine-cystine, leucine, lysine or arginine deficiency. Chicks fed diets deficient in these amino acids gained more weight on less feed than those fed diets deficient in all amino acids. Also, despite the known positive association between feed consumption and feed efficiency in young chicks, a reduction in feed consumption of over 30% by chicks consuming tryptophan- or phenylalanine plus tyrosine-deficient diets failed to depress gain-to-feed ratios below that of 60% RS (table 3). Perhaps chicks fed 60% RS contained more fat and less protein in their gain than those fed diets deficient in a single amino acid.

The data resulting from these experiments support the view that the pattern of amino acids in excess of that needed for protein synthesis dictates whether the effect of an amino acid imbalance will be superimposed on that of an amino acid deficiency. This could explain why diets equally deficient in a single amino acid do not necessarily result in equal gains. The disparities noted between bioassay and "chemical score" could result from failure of the latter to consider the qualitative and quantitative differences in the pattern of excess amino acids.

LITERATURE CITED

1. Huston, R. L., and H. M. Scott 1968 Effect of varying the composition of a crystalline amino acid mixture on weight gain and pattern of free amino acids in chick tissue. Federation Proc., 27: 1204.
2. Dean, W. F., and H. M. Scott 1965 The development of an amino acid reference diet for the early growth of chicks. Poultry Sci., 44: 803.
3. Leveille, G. A., R. Shapiro and H. Fisher 1960 Amino acid requirements for maintenance in the adult rooster. IV. The requirements for methionine, cystine, phenylalanine, tyrosine and tryptophan; the adequacy of the determined requirements. J. Nutr., 72: 8.

Effect of Dietary Fat Source on the Apparent Digestibility of Fat and the Composition of Fecal Lipids of the Young Pig [1,2]

R. M. G. HAMILTON [3] AND B. E. McDONALD [4]
Department of Animal Science, Macdonald College (McGill University), Montreal, Quebec, Canada

ABSTRACT Two experiments, each of 5 weeks' duration, were conducted with 18-day-old weanling pigs to determine the effect of dietary fat source and age of animal on apparent fat digestion and absorption. In each experiment two males and two females were fed diets that contained 10% coconut oil, rapeseed oil, lard or tallow. Fecal collections were made for 5 consecutive days beginning at 21 and 49 days of age. The petroleum ether-soluble lipids of the diets and of acidified (HCl-ethanol) lyophilized feces were extracted and fractionated into free fatty acids (FFA), fatty acids of glyceryl esters (FAGE) and nonsaponifiables using a 0.05 N KOH-60% ethanol system. None of the differences in average percent digestibility of the dietary fat, which varied from 63.8 to 78.4 at 21 days of age and from 69.6 to 82.2 at 49 days of age, was related to fat source, sex of animals or age of the animals. Partition of the fecal lipids revealed that the FFA fraction accounted for 74 to 85%, and the FAGE fraction, 4 to 8%, of the total lipids excreted by the pig, irrespective of fat source or age of the animal, which suggests that digestion (that is, hydrolysis) of the dietary fat was essentially complete at both 21 and 49 days of age. Gas-liquid chromatographic analyses indicated that dietary fatty acid composition influenced the fatty acid patterns of the fecal lipids, although there were notable qualitative and quantitative differences. Total saturated fatty acids constituted a higher proportion of the FFA fraction than of the dietary lipid fraction or of the FAGE fraction for groups fed rapeseed oil, lard or tallow. Apparent digestibilities of palmitic and stearic acids were lower than those of the unsaturated or medium-chain saturated fatty acids.

The weanling pig, like the young of many species, has been reported to utilize dietary fat inefficiently (1–3). Lloyd and Crampton (1) observed an inverse relationship between molecular weight of dietary fat and its apparent digestibility. Fat utilization by the young pig, however, has been reported to improve rapidly with the age of the animal (2, 3). Furthermore, Lloyd et al. (2) reported that the improvement in apparent fat digestibility with age of the animal was most marked for the high molecular-weight fats.

Kitts et al. (4) and Hartman et al. (5) reported that pancreatic lipase activity of the suckling pig was relatively high at birth and increased only slightly with age. Hartman et al. (5), however, observed that pancreatic tributyrinase activity of pigs weaned at 7 days of age decreased following weaning and did not return to the level observed in comparable pigs suckling until 6 to 7 weeks of age. These authors postulate that this postweaning decrease in tributyrinase activity may explain in part the observed improvement in apparent fat digestibility with age.

The studies reported here were conducted to determine the effects of dietary fat source and age of the animal on the composition of fecal lipids and on the apparent digestibility of fat by the weanling pig.

EXPERIMENTAL

Animal trials. Thirty-two weanling pigs, two replicates of eight males and eight females, were used in the investigation. The

Received for publication August 12, 1968.

[1] From a thesis by R. M. G. Hamilton, submitted in partial fulfillment of the requirements for the Master of Science degree, McGill University, Montreal, Quebec, Canada.

[2] A report on this work presented at the 11th Annual Meeting of the Canadian Federation of Biological Societies, Kingston, Ontario, Canada, June, 1968. Supported in part by National Research Council of Canada Grant no. A-3262. R. M. G. Hamilton received financial support from a National Research Council postgraduate scholarship.

[3] Present address: Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada.

[4] Present address: School of Home Economics, University of Manitoba, Winnipeg, Manitoba, Canada.

pigs were weaned at approximately 18 days of age and immediately assigned to treatment groups. Animals were randomly allotted to groups of two males or two females with the restriction that littermates were distributed across treatments. All pigs had access to a commercial baby pig diet [5] during the nursing period, and all males were orchiectomized at 7 days of age.

Each pair of animals was housed in a pen (1.3 m by 2.6 m) with a solid concrete floor. Room temperature was maintained at 22° with auxiliary heat provided in each pen by an infrared heat lamp. Feed (composition in table 1) and water were supplied ad libitum.

Digestibility determinations. Apparent digestibility coefficients [6] were determined by the chromium sesquioxide indicator method. Feces excreted by each pair of animals were collected twice during a 6-hour period (8:00 AM to 2:00 PM) for 5 consecutive days beginning at 21 and 49 days of age. All fecal samples were immediately stored at −15°. At the time of analysis, the daily fecal collections for each group were pooled and mixed; and a 100-g aliquot was lyophilized for 16 hours at 0.5 to 1.0 mm Hg, using dry ice-acetone as a refrigerant.

Feed and feces were analyzed for dry matter and nitrogen by the AOAC method (7) and for chromic oxide by the method of Christian and Coup (8).

TABLE 1

Composition of diets

	% of diet
Wheat	16.42
Oat groats	30.00
Soybean meal (50% protein)	23.00
Dried brewer's yeast	5.00
Dried skim milk (roller processed)	11.00
Ground limestone	1.30
Dicalcium phosphate	2.00
Methionine hydroxy analogue, calcium [1]	0.28
Butylated hydroxy toluene [1]	0.10
Antibiotic supplement [2]	0.10
Vitamin-mineral premix [3]	0.55
Chromium sesquioxide	0.25
Fat (coconut oil, rapeseed oil, lard or tallow) [4]	10.00

[1] Monsanto Canada Ltd., Montreal, Canada.

[2] Provided per kilogram diet: 44 mg chlortetracycline, 44 mg sulfamethazine, and 22 mg penicillin. Cyanamid of Canada Ltd., Montreal, Canada.

[3] Provided per kilogram diet: vitamin A, 1580 IU; vitamin D_3, 200 IU; riboflavin, 480 μg; vitamin B_{12}, 17 μg; salt, 4.9 g; and zinc sulfate heptahydrate, 79.4 mg.

[4] Lard and tallow were donated by Canadian Renderers Association, Toronto, Canada. Coconut oil and rapeseed oil were purchased from Canada Packers Ltd., Montreal, Canada.

Lipid analysis. Lipids were extracted from a 1.0- to 2.0-g sample of feed or acidified lyophilized feces according to method B of van der Kamer et al. (9). The extracted lipids (100 to 200 mg) were partitioned into free fatty acids (FFA) and neutral lipids by the procedure of Borgström (10), except that the alkaline ethanol solvent contained ethanol–KOH–amyl or iso-amyl alcohol–distilled water (60:5:0.4:34.6, v/v/v/v). The neutral lipid fractions were further partitioned into fatty acids from glyceryl esters (FAGE) and nonsaponifiables (NSF) by the method of van de Kamer (11). Volumes of solvents, however, were one-half those specified by van de Kamer, except for washing of the NSF layer (20 ml 75% ethanol was used) and washing of the FAGE layer (40 ml 1 N HCl in 25% ethanol was used). Fatty acid methyl esters were prepared from the FFA and FAGE fractions using boron trifluoride at the catalyst (12).

The fatty acid esters were resolved with stainless steel columns (2.7 m by 2 mm, interior diameter) packed with 10% EGSS-Y organosilicone polyester coated on 100/200-mesh Gas Chrom Q,[7] using a gas chromatograph [8] fitted with dual columns and hydrogen flame detectors. Helium flow rates were 30 to 35 ml/minute. For coconut-oil samples, the temperature program was 120 to 195° at 8°/minute, with the injection port at 150° and detectors at 135°. For rapeseed oil, lard and tallow samples, the temperature program was 160 to 195° at 2°/minute with injection port and detectors at 180°. Column temperature was held isothermal for 1 minute before commencement of the temperature program. Identification of the fatty acid methyl esters was made by comparison with known standards.[9] The area under the peaks for the individual fatty acids was measured

[5] Supersweet Pre-Creep Pig Starter, Robin Hood Flour Mills Ltd., (Supersweet Feeds Division), Montreal, Quebec, Canada.

[6] Apparent digestibility, as used in this paper, expresses the difference between the amount of a nutrient ingested and the amount excreted in the feces relative to the amount ingested (6).

[7] Applied Science Laboratories, Incorporated, State College, Pennsylvania.

[8] Model 881, Perkin-Elmer Corporation, Norwalk, Connecticut.

[9] The Hormel Institute, Austin, Minnesota.

by a printing integrator [10] and corrected for changes in the base line.

Statistical analysis. The results were subjected to analysis of variance (13).

RESULTS

Dietary fat source did not affect ($P < 0.05$) average daily weight gain, feed/gain ratio or apparent digestibility of protein, dry matter or fat (table 2). The apparent digestibilities of crude protein, dry matter and total lipid, however, tended to improve with the age of the animals; the apparent digestibility of crude protein and dry matter was significantly higher ($P < 0.05$) at 49 days than at 21 days. In general, the improvement in the digestibility coefficients for crude protein and dry matter between 21 and 49 days of age was greater for males than females. This may partially explain the slightly higher rate of gain exhibited by the males, although there was no significant sex-age interaction. Differences in the apparent digestibility of the lipid fraction due to dietary fat source, age or sex were not significant ($P < 0.05$).

The intake and excretion patterns of the individual fatty acids for pigs fed the different fat sources are summarized in table 3. Apparent digestibility was relatively high for all fatty acids, except stearic acid, and appeared to be independent of dietary fat source or age of the animals. Apparent digestibility of the saturated fatty acids, however, tended to decrease as fatty acid chain length increased, although the relationship was confounded to some extent by the concentration of the various fatty acids in the diet. Comparison of the intake and excretion patterns of myristic acid and stearic acid illustrates the effect of chain length on apparent digestibility of the saturated fatty acids. The apparent digestibility of myristic acid was relatively high for all groups irrespective of dietary level. The apparent digestibility of stearic acid was, however, relatively low for animals fed diets containing lard or tallow; stearic acid excretion was greater than its ingestion for animals fed diets containing coconut oil or rapeseed oil. The relationship between chain length and apparent digestibility coefficients for the monounsaturated fatty acids was not as definitive (rapeseed oil, table 3).

Utilization of the long-chain saturated fatty acids tended to improve with the age of the animal. This is shown by the higher apparent digestibilities of palmitic acid and stearic acid at 49 days of age for the groups fed coconut oil, rapeseed oil and tallow. The data in table 3 also illustrate the effect of steric position in the triglyceride molecule on the apparent digestibility of long-chain saturated fatty acids. The amount

[10] Model 194B, Perkin-Elmer Corporation, Norwalk, Connecticut.

TABLE 2

Summary of average daily gain, feed/gain ratio and apparent digestibility

	Age	Dietary fat							
		Coconut oil		Rapeseed oil		Lard		Tallow	
		Male	Female	Male	Female	Male	Female	Male	Female
	days								
Avg initial wt, kg		5.55	5.56	5.85	5.39	5.18	4.94	5.35	5.48
Avg daily gain, g		438	394	425	369	404	372	437	377
Feed/gain		1.36	1.37	1.42	1.37	1.38	1.45	1.34	1.42
		Apparent digestibility, % [1]							
Crude protein	21	77.8	80.4	78.1	81.1	78.6	76.0	75.3	77.8
	49	86.0	82.1	85.5	86.4	84.1	82.4	85.0	83.4
Dry matter	21	81.9	84.5	82.6	84.1	83.8	82.2	80.5	82.2
	49	86.9	83.8	86.6	86.1	85.9	83.2	85.2	84.0
Total lipid	21	71.5	78.4	77.5	74.8	77.1	68.7	63.8	68.1
	49	79.8	80.5	82.2	77.3	77.6	74.8	74.2	69.6

[1] Based on composite samples of feces collected for 5 consecutive days beginning at average ages of 21 and 49 days.

TABLE 3

Dietary intake, fecal excretion and apparent digestibility of individual fatty acids

Fatty acid [1]	Coconut oil				Rapeseed oil				Lard				Tallow			
	Intake [2]	Excretion [2]		Coef [3] app dig	Intake	Excretion		Coef app dig	Intake	Excretion		Coef app dig	Intake	Excretion		Coef app dig
		Male	Female			Male	Female			Male	Female			Male	Female	
	mg fatty acid/mg chromic acid			%	*mg fatty acid/mg chromic acid*			%	*mg fatty acid/mg chromic acid*			%	*mg fatty acid/mg chromic acid*			%
							21 days of age [4]									
$C_{10:0}$	2.0	—	—	100												
$C_{12:0}$	15.8	1.5	0.7	95												
$C_{14:0}$	6.4	0.4	0.8	90					0.6	0.2	0.1	75	1.5	0.2	0.1	90
$C_{16:0}$	3.1	2.6	1.9	27	2.4	0.8	1.2	59	12.4	2.2	3.9	75	11.4	4.7	4.3	60
$C_{18:0}$	0.8	3.1	1.4	−179	0.7	2.2	2.0	−208	8.1	3.4	5.9	42	6.1	5.4	4.3	21
$C_{18:1}$	2.8	1.6	1.4	44	11.0	1.3	1.5	88	16.3	1.6	1.9	89	17.7	2.4	2.2	87
$C_{18:2}$	0.8	0.5	0.6	30	7.6	0.3	0.4	96	4.3	0.4	0.3	91	2.0	1.0	0.5	68
$C_{18:3}$					3.5	—	—	100								
$C_{20:1}$					5.0	0.3	0.3	94								
$C_{22:1}$					8.6	1.8	2.7	74								
							49 days of age [5]									
$C_{10:0}$	1.8	—	—	100												
$C_{12:0}$	14.9	0.8	1.0	94												
$C_{14:0}$	6.0	0.7	0.4	90					0.6	< 0.1	< 0.1	92	1.5	0.1	0.2	90
$C_{16:0}$	3.0	1.5	1.2	54	2.4	0.6	0.7	73	11.8	2.1	2.5	81	11.4	3.3	3.8	67
$C_{18:0}$	0.8	1.0	0.6	0	0.7	0.7	1.0	−21	7.7	3.6	4.1	50	6.0	3.5	3.9	30
$C_{18:1}$	2.6	1.3	1.5	46	10.8	1.4	1.8	85	15.5	2.1	1.9	87	17.5	2.0	2.2	88
$C_{18:2}$	0.7	0.5	0.8	6	7.5	0.6	0.6	93	4.1	0.4	0.4	90	2.0	0.4	0.5	88
$C_{18:3}$					3.5	—	—	100								
$C_{20:1}$					4.9	0.3	0.6	91								
$C_{22:1}$					8.5	1.9	2.4	75								

[1] Number of carbon atoms:number of double bonds.
[2] Intake and excretion expressed as milligrams fatty acid per milligrams chromic oxide in diet and feces, respectively.
[3] Mean coefficient of apparent digestibility for males and females.
[4] Based on composite samples of feces collected for 5 consecutive days beginning at an average age of 21 days.
[5] Based on composite samples of feces collected for 5 consecutive days beginning at an average age of 49 days.

of palmitic acid excreted by pigs fed the tallow diet was considerably higher than by the pigs fed the lard diet, although the concentration of palmitic acid was similar in these diets.

Partition of the fecal lipids (table 4) showed free fatty acids (FFA) to be the major constituent irrespective of dietary fat source, age or sex. The FFA accounted for 74 to 85% of the total fecal lipid excreted by the pigs, whereas the fatty acids of glyceryl esters (FAGE) accounted for only 4.3 to 7.9% of the total lipid excreted. Not only did the FAGE fraction constitute a relatively small proportion of the total fecal lipid, but also resolution of the extracted fecal lipid by thin-layer chromatography (fig. 1) showed only trace amounts of material with R_F values similar to that of tristearin. There was no significant difference in the percentage composition of the fecal lipids due to dietary fat source, sex or age.

The fatty acid patterns of the FAGE and FFA fractions resembled those of the dietary lipids, although there were a number of notable differences (table 5). The proportion of stearic acid, for example, was much higher in the fecal lipids than in the feed, whereas the percentage of lauric acid was higher in feed than feces. Linoleic acid constituted 9.0% of the total fatty acids in the rapeseed oil diet, although no measurable quantity of this fatty acid was present in the fecal lipids. By contrast, arachidic acid and behenic acid were present in the FAGE and FFA fractions of the fecal lipids from these animals, although neither occurred in the diet.

There was an appreciable difference in the percentage fatty acid composition of the FAGE and FFA fraction. The level of oleic acid was higher in the FAGE fraction than the FFA fraction, with the exception of the 49-day collection for the group fed rapeseed oil, whereas the level of stearic acid was higher in the FFA fraction than in the FAGE fraction. There was no appreciable difference, however, in the fatty acid composition of the fecal lipid fractions for the 21- and 49-day collection periods.

TABLE 4

Effect of dietary fat source and age on composition of fecal lipids

Dietary fat	Sex	21 days of age				49 days of age			
		Total lipid in feces	Composition of fecal lipids			Total lipid in feces	Composition of fecal lipids		
			FAGE [1]	FFA [2]	NSF [3]		FAGE	FFA	NSF
		mg/g [4]	%	%	%	*mg/g*	%	%	%
Coconut oil	male	179.0 ± 5.7 [5]	5.2 ± 0.9	80.8 ± 4.9	12.9 ± 1.7	136.3 ± 2.9	4.5 ± 0.7	79.4 ± 0.8	11.1 ± 0.2
	female	147.7 ± 2.4	5.5 ± 1.7	78.5 ± 0.6	11.7 ± 1.4	136.3 ± 11.1	5.6 ± 1.7	77.0 ± 5.7	11.8 ± 2.3
Rapeseed oil	male	148.8 ± 4.0	6.8 ± 0.2	79.0 ± 2.1	10.9 ± 1.9	154.3 ± 24.9	4.8 ± 0.3	75.9 ± 2.0	13.5 ± 0.9
	female	179.9 ± 26.8	6.1 ± 1.4	78.5 ± 4.7	10.4 ± 1.3	187.6 ± 7.7	4.6 ± 0.7	75.2 ± 9.6	11.3 ± 2.1
Lard	male	171.8 ± 18.0	7.9 ± 4.2	73.8 ± 1.9	16.0 ± 3.7	185.3 ± 3.6	4.3 ± 0.4	84.5 ± 0.3	7.9 ± 1.1
	female	220.6 ± 13.2	5.0 ± 1.3	85.0 ± 5.0	10.7 ± 1.0	181.9 ± 27.2	5.0 ± 0.1	80.8 ± 5.3	11.1 ± 1.3
Tallow	male	219.8 ± 64.8	5.2 ± 0.2	81.7 ± 1.6	9.3 ± 0.7	204.2 ± 25.5	4.4 ± 0.8	84.7 ± 3.2	8.5 ± 1.6
	female	212.5 ± 46.1	5.3 ± 0.1	82.3 ± 2.7	9.4 ± 2.5	217.7 ± 31.3	4.5 ± 0.8	83.4 ± 2.6	8.1 ± 0.8
	Mean value [6]		5.9 ± 1.0	80.0 ± 3.3	11.4 ± 2.2		4.7 ± 4.2	80.1 ± 3.8	10.4 ± 2.0

[1] Fatty acids of glyceryl esters.
[2] Free fatty acids.
[3] Nonsaponifiable fraction.
[4] Expressed on a dry matter basis.
[5] Mean ± $SD_{\bar{x}}$.
[6] Mean ± $SD_{\bar{x}}$ for all fat sources.

DISCUSSION

Improvement in the apparent digestibilities of crude protein and dry matter be-

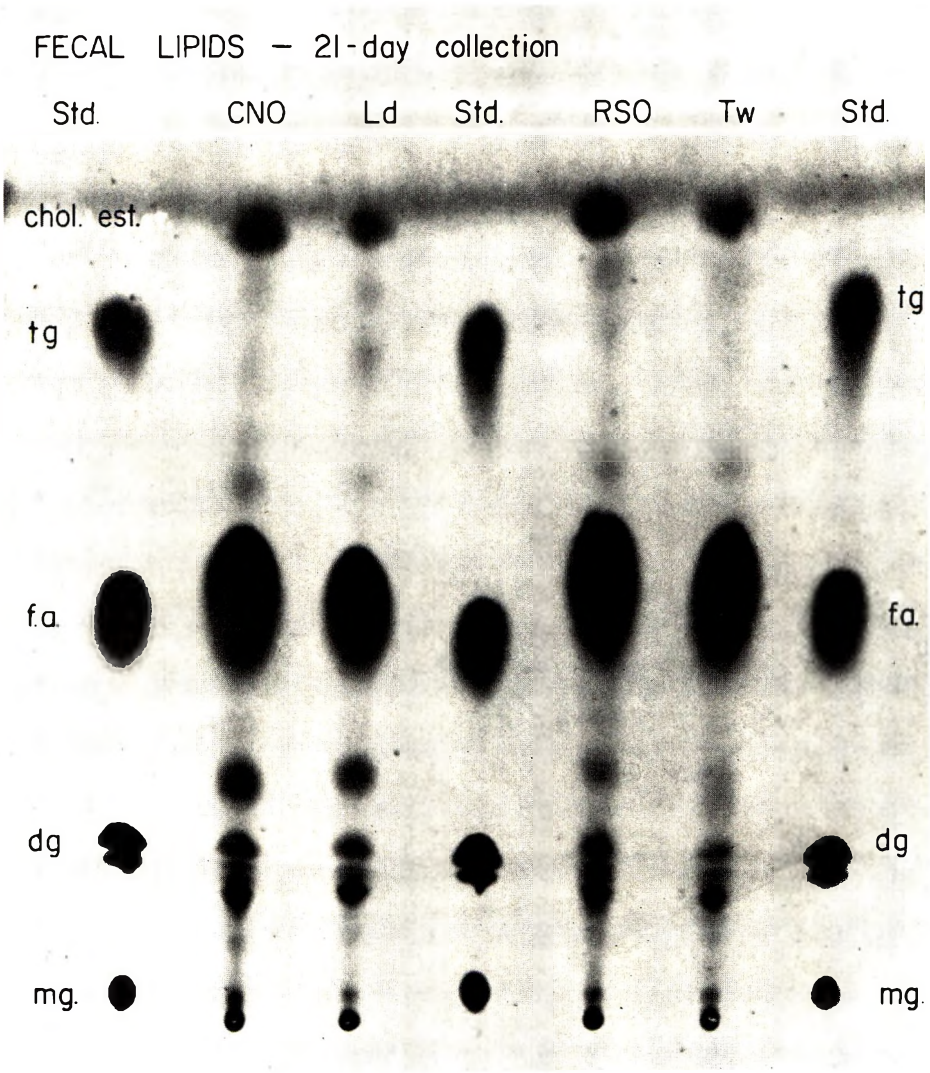


Fig. 1 A thin-layer chromatographic comparison of the composition of the extracted fecal lipids from 21-day-old animals fed diets that contained coconut oil (CNO), lard (Ld), rapeseed oil (RSO) or tallow (Tw). Standard (Std) contained monopalmitin (mg), distearin (dg), palmitic acid (fa) and tristearin (tg). (Components in Std were obtained from The Hormel Institute, Austin, Minnesota.)

tween 21 and 49 days of age agrees with previously reported data for the young pig (2, 3). The failure to find any significant changes in the apparent digestibility of fat with age, however, does not agree with the results of Lloyd et al. (2) and Eusebio et al. (3), who reported significant improvements in fat digestibility by the pig between 3 and 7 weeks of age. This discrepancy was due primarily to a higher apparent fat digestibility at 21 days of age in the present study. Part of the difference between these studies may be associated with the preexperimental management of the animals. Hartman et al. (5) found that there was a transitory decrease in lipase activity when young pigs were abruptly weaned to dry diets, while Boyd and Edwards (14) reported significantly higher fat digestibility values with germfree chicks than with conventional chicks. The pigs used in the present study had access to a commercial

TABLE 5

Percentage fatty acid composition of dietary fat and fecal lipid fractions

Fatty acid [1]	Coconut oil			Rapeseed oil			Lard			Tallow		
	Feed	Feces		Feed	Feces		Feed	Feces		Feed	Feces	
		FAGE [2]	FFA [3]		FAGE	FFA		FAGE	FFA		FAGE	FFA
					21 days of age [4]							
$C_{10:0}$	5.6	— [5]	—									
$C_{12:0}$	47.6	15.0 ± 1.4	9.6 ± 1.3				tr	tr	tr	tr	tr	tr
$C_{14:0}$	19.2	9.7 ± 1.2	10.9 ± 0.4	tr [6]	tr	tr	1.5	3.1 ± 2.3	2.8 ± 2.6	3.9	1.6 ± 0.3	1.2 ± 0.2
$C_{14:1}$	—	4.8 ± 1.3	0.7 ± 0	—	1.8 ± 0.4	tr	—	4.1 ± 1.5	1.4 ± 0	tr	3.8 ± 0.6	tr
$C_{16:0}$	10.4	18.2 ± 1.5	27.8 ± 2.6	6.3	11.2 ± 1.4	12.5 ± 1.8	29.7	22.2 ± 2.1	29.8 ± 2.2	29.6	22.4 ± 2.3	36.6 ± 1.5
$C_{18:0}$	2.4	7.8 ± 2.0	27.2 ± 7.7	1.7	9.1 ± 4.8	25.5 ± 6.2	19.4	14.1 ± 5.0	47.3 ± 3.5	15.7	17.4 ± 6.5	38.4 ± 5.2
$C_{18:1}$	8.3	23.2 ± 4.2	18.6 ± 2.4	28.3	23.7 ± 2.7	17.1 ± 4.7	39.0	29.9 ± 6.6	16.3 ± 3.2	45.7	34.7 ± 5.2	15.3 ± 7.9
$C_{18:2}$	2.3	18.6 ± 2.7	6.0 ± 2.2	19.6	11.1 ± 2.2	3.8 ± 1.8	10.4	17.6 ± 5.4	2.5 ± 1.0	5.1	14.5 ± 6.2	3.6 ± 2.3
$C_{18:3}$				9.0	—	—	tr	2.3 ± 0.4	1.3 ± 0.1	tr	1.7 ± 0.5	tr
$C_{20:0}$				—	2.3 ± 0.7	3.2 ± 1.8				—		
$C_{20:1}$				12.8	5.7 ± 1.1	3.9 ± 1.0				—	1.7 ± 0.7	—
$C_{22:0}$				—	5.1 ± 3.0	6.7 ± 4.1						
$C_{22:1}$				22.3	31.9 ± 5.8	27.2 ± 5.4						
					49 days of age [7]							
$C_{10:0}$	5.6	—	—									
$C_{12:0}$	47.6	10.0 ± 1.5	14.3 ± 2.7	—			tr	tr	tr	tr	tr	tr
$C_{14:0}$	19.2	6.8 ± 3.5	11.9 ± 0.5	tr	tr	tr	1.5	1.1 ± 0.1	0.9 ± 0	3.9	1.9 ± 0.6	1.5 ± 0.2
$C_{14:1}$	—	5.6 ± 1.5	0.8 ± 0.2	—	3.1 ± 1.8	tr	—	4.1 ± 1.3	tr	tr	4.0 ± 1.2	tr
$C_{16:0}$	10.4	19.0 ± 5.0	21.6 ± 7.0	6.3	11.2 ± 1.8	10.0 ± 1.2	29.7	23.6 ± 1.2	26.7 ± 2.1	29.6	26.5 ± 2.4	35.8 ± 1.4
$C_{18:0}$	2.4	5.6 ± 2.0	13.6 ± 6.9	1.7	8.4 ± 3.7	12.8 ± 2.3	19.4	13.7 ± 4.1	46.0 ± 4.5	15.7	19.8 ± 1.4	37.1 ± 7.3
$C_{18:1}$	8.3	25.0 ± 1.4	22.5 ± 1.1	28.3	23.9 ± 1.9	24.9 ± 2.7	39.0	35.0 ± 2.1	23.3 ± 4.7	45.7	32.7 ± 4.9	20.1 ± 3.6
$C_{18:2}$	2.3	21.0 ± 4.4	9.7 ± 4.2	19.6	11.0 ± 0.8	8.5 ± 1.8	10.4	19.9 ± 5.2	4.0 ± 0.8	5.1	10.4 ± 1.4	3.8 ± 1.9
$C_{18:3}$				9.0	—	—	tr	tr	tr	tr	1.4 ± 0.3	tr
$C_{20:0}$				—	2.1 ± 0.6	2.6 ± 1.1				—		
$C_{20:1}$				12.8	6.3 ± 2.0	5.5 ± 2.1				—		
$C_{22:0}$				—	1.8 ± 0.5	2.2 ± 0.4						
$C_{22:1}$				22.3	31.9 ± 6.9	31.7 ± 5.3						

[1] Number of carbon atoms:number of double bonds.
[2] Fatty acids of glyceryl esters.
[3] Free fatty acids.
[4] Based on composite samples of feces collected for 5 consecutive days beginning at an average age of 21 days.
[5] No measurable amounts detected.
[6] Trace, < 0.5% of total fatty acids.
[7] Based on composite samples of feces collected for 5 consecutive days beginning at an average age of 49 days.

baby pig diet which contained an antibiotic supplement from 7 days of age until the start of the experiment. Another factor which might affect fat digestibility determinations is the method used to extract the fecal lipids. A petroleum ether-HCl system was used by the authors, whereas Lloyd et al. (2) and Eusebio et al. (3) employed a single neutral diethyl ether extraction. Extraction of feces with neutral diethyl ether or petroleum ether removes only a portion of the soaps (15), and reextraction of the fecal residue after acidification removes additional lipid (15, 16).

Intake and excretion data for the individual fatty acids showed palmitic and stearic acids to be poorly utilized by the young pig. The weanling pig did not utilize stearic acid as efficiently as unsaturated fatty acids of the same chain length; this agrees with previously reported results for the rat (15), the pig (16) and the chicken (17, 18). Low apparent digestibility coefficients for palmitic and stearic acids for the pigs fed diets that contained lard or tallow may be partly due to the excretion of metabolic fecal lipids containing a high proportion of these fatty acids, because animals fed the coconut oil or rapeseed oil diets excreted more stearic acid than was ingested. Digestibilities of palmitic and stearic acids for the lard and tallow diets, however, were still low, even when allowance was made for metabolic fecal lipids on the basis of the excretion patterns for the coconut oil or rapeseed oil diets.

Poor utilization of the long-chain saturated fatty acids appears to be associated with faulty absorption of these fatty acids. Support for this postulate is contained in the apparent digestibility data for palmitic and stearic acids, which were higher for lard than for tallow. Similar data have been reported for the chicken (17). Since palmitic acid is preferentially esterified at the 2-position in lard (19) and lipase specifically hydrolyzes the 1- and 3-ester bonds of triglycerides (20), much of the palmitic acid in lard would be absorbed as the 2-monopalmitin. The peculiar steric configuration of lard may also account for the higher apparent digestibility of stearic acid from this fat. Young and Garrett (21) have reported that the absorption of palmitic and stearic acids by chicks fed free fatty acids was influenced not only by the ratio of saturated to unsaturated fatty acids but also by the ratio of free palmitic to free stearic acid in the diet. Faulty absorption also is implicated by the fact that palmitic and stearic acids occurred primarily as free fatty acids in the fecal lipids.

The high proportions of FFA in the fecal lipids, together with the low levels of FAGE and the detection of only trace amounts of triglyceride, coincide with the observations that lipase activity is relatively high in the young pig (4, 5). High proportions of FFA in the fecal lipids have also been reported for the human (9, 22).

The fatty acid patterns of the fecal lipid fractions resembled those of the dietary fat sources, although there were a number of important qualitative and quantitative differences. Total saturated fatty acids constituted a higher proportion of the FFA fraction, for example, than of either the dietary lipid or the FAGE fraction for groups fed rapeseed oil, lard or tallow. The presence of arachidic and behenic acids in the fecal lipid fractions of animals fed the rapeseed oil diet, although neither was present in the diet, further illustrates the differences between fecal and dietary fatty acid patterns. Arachidic and behenic acids could arise through hydrogenation of eicosenoic and erucic acids by intestinal microflora or by enzymes liberated from sloughed-off mucosal cells, although Bloch et al. (23) have suggested that there is considerable resistance to the enzymatic reduction of the double bonds of unsaturated fatty acids.

The low levels of palmitoleic acid in the dietary fat sources used in this study, with the suggestion by Bloch et al. (23) that unsaturated fatty acids are resistant to hydrogenation by biological systems, suggest that the high levels of palmitic and stearic acids in the fecal lipids of the pig represent faulty absorption rather than hydrogenation of unsaturated homologues of these fatty acids.

ACKNOWLEDGMENT

The authors express their appreciation to K. R. Bruce for assistance with the chemical analysis.

LITERATURE CITED

1. Lloyd, L. E., and E. W. Crampton 1957 The relation between certain characteristics of fats and oils and their apparent digestibility by young pigs, young guinea pigs and pups. J. Anim. Sci., *16:* 377.
2. Lloyd, L. E., E. W. Crampton and V. G. MacKay 1957 The digestibility of ration nutrients by three- vs. seven-week old pigs. J. Anim. Sci., *16:* 383.
3. Eusebio, J. A., V. W. Hays, V. C. Speer and J. T. McCall 1965 Utilization of fat by young pigs. J. Anim. Sci., *24:* 1001.
4. Kitts, W. D., C. B. Bailey and A. J. Wood 1956 The development of the digestive enzyme system of the pig during its preweaning phase of growth. A. Pancreatic amylase and lipase. Can. J. Agr. Sci., *36:* 45.
5. Hartman, P. A., V. W. Hays, R. O. Baker, L. H. Neagle and D. V. Catron 1961 Digestive enzyme development in the young pig. J. Anim. Sci., *20:* 114.
6. Deuel, H. J., Jr. 1955 The Lipids, vol. 2. Interscience Publishers, Inc., New York.
7. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
8. Christian, K. R., and M. R. Coup 1954 The determination of chromic oxide in faeces. New Zeal. J. Sci. Technol., *36:* 328.
9. Van der Kamer, J. H., H. ten Bokkel Huinink and H. A. Weyers 1949 Rapid method for the determination of fat in feces. J. Biol. Chem., *177:* 347.
10. Borgström, B. 1952 Investigation of lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. Acta Physiol. Scand., *25:* 111.
11. Van de Kamer, J. H. 1953 Quantitative determination of the saturated and unsaturated higher fatty acids in fecal fat. Scand. J. Clin. Lab. Invest., *5:* 30.
12. Metcalfe, L. D., A. A. Schmitz and J. R. Pelka 1966 Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem., *38:* 514.
13. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., Toronto, Ontario.
14. Boyd, F. M., and H. M. Edwards, Jr. 1967 Fat absorption by germ-free chicks. Poultry Sci., *46:* 1481.
15. Carroll, K. K. 1958 Digestibility of individual fatty acids in the rat. J. Nutr., *64:* 399.
16. Bayley, H. S., and D. Lewis 1965 The use of fats in pig feeding. II. The digestibility of various fats and fatty acids. J. Agr. Sci., *64:* 373.
17. Renner, R., and F. W. Hill 1961 Utilization of fatty acids by the chicken. J. Nutr., *74:* 259.
18. Young, R. J. 1961 The energy value of fats and fatty acids for chicks. I. Metabolizable energy. Poultry Sci., *40:* 1225.
19. Mattson, F. H., R. A. Volpenhein and E. S. Sutton 1964 The distribution of fatty acids in the triglycerides of the Artiodactyla (eventoed animals). J. Lipid Res., *5:* 363.
20. Senior, J. R. 1964 Intestinal absorption of fats. J. Lipid Res., *5:* 495.
21. Young, R. J., and R. L. Garrett 1963 Effect of oleic and linoleic acids on the absorption of saturated fatty acids in the chick. J. Nutr., *81:* 321.
22. Fowweather, F. S., and W. N. Anderson 1946 A method for the determination of fat in feces. Biochem. J., *40:* 350.
23. Bloch, K., P. Baronowsky, H. Goldfine, W. J. Lennarz, R. Light, A. T. Norris and G. Scheuerbrandt 1961 Biosynthesis and metabolism of unsaturated fatty acids. Federation Proc., *20:* 921.

Neural Lesions in the Offspring of Female Rats Fed a Copper-deficient Diet [1,2,3]

WILLIAM W. CARLTON AND WILLIAM A. KELLY
Department of Veterinary Physiology and Pharmacology, School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana

ABSTRACT Weanling female rats of the Holtzman strain fed a copper-deficient diet (1 ppm) were mated at sexual maturity to normal males. Offspring were continued on this same diet. Within 6 weeks, some presented signs of neurologic dysfunction such as hyperirritability after noise stimulation, catatonic postures, convulsive seizures and, in male rats, priapism. Gross lesions consisted of focal pale areas in various regions of the cerebral cortex. Pathoanatomic alterations were observed in the brain with the cerebral cortex and corpus striatum consistently affected, but lesions were also present in the thalamic region of the diencephalon. Neural tissue was rarefied, spongy, edematous and obviously necrotic. Reactions to the necrosis included capillary proliferation and hemorrhage, but a significant astrocytosis was not detected. Swelling of endothelium and hyperplasia of pericytes were observed. In severely affected animals hemorrhage and liquefactive necrosis were accompanied by status spongiosus of surrounding tissue. The lesions are consistent with alterations produced by severe tissue hypoxia.

A disease of the nervous system of young lambs was described by Bennetts (1) in Australia and called enzootic ataxia. A disease with similar clinical and morphological features was described by Innes in 1936 (2). Clinical signs included spastic paralysis of the hind limbs, ataxia and a progressive course to a fatal termination. Neural lesions considered characteristic included a diffuse symmetrical degeneration of the cerebral white matter and secondary degeneration of the motor tracts into the spinal cord. "Swayback" as the disease was called in England was studied by Innes and Shearer (3); they found that the brains of severely affected ataxic newborn lambs had massive collapsed areas in the cerebral hemispheres. Large cavitations were observed when the brains were sectioned. Enzootic ataxia and swayback can be prevented by administration of copper to pregnant ewes (4–6).

The view that swayback was essentially a demyelinating disease as suggested by Innes and Shearer (3) was challenged by Behrens and von Schulz (7, 8) who suggested that the lesions resulted from venous stasis, edema and perivascular necrosis. Other investigators (9) agreed with this viewpoint of the pathogenesis of the encephalopathy.

Barlow and co-workers (10–12), though in agreement that the cerebral lesions are pathognomonic for swayback when present, reported that these changes need not be the essential feature of the disease as over half of a series of affected lambs did not have these cerebral lesions. These authors regarded scattered lesions of hyaline necrosis of neurons and nerve fiber degeneration in the brain stem and spinal cord as the essential pathologic alterations. Barlow (13) described the neural lesions in the brains of several age groups and suggested that the disease process continues after birth with the spinal cord particularly involved; nerve fiber changes were constantly observed in the lateral funiculus adjacent to the dorsal root in sheep surviving for several weeks to several months.

Mills and Fell (14) observed degenerative changes in the motor neurons of the red nucleus and ventral horns of the gray matter in the spinal cord of lambs from

Received for publication July 29, 1968.
[1] Submitted as Journal paper 3432 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana.
[2] Supported in part by Contract no. 12-14-100-8908(61) from the Human Nutrition Research Division ARS, USDA, Beltsville, Maryland.
[3] Presented in part at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 15–20, 1968.

ewes fed a diet high in sulfate and molybdate. Demyelination occurred in the cerebral cortex and spinal cord. Under somewhat different experimental conditions, Butler and Barlow (15) could not confirm the results of Mills and Fell (14) as the administration of large amounts of molybdate and sulfate to pregnant Blackface ewes did not produce swayback in their lambs.

An unusual nervous disease of lambs described by Roberts et al. (16, 17) was characterized by severe brain swelling accompanied by the microscopic changes of cerebral edema, cortical necrosis, neuronal degeneration and spinal cord lesions typical of delayed swayback. A constant profound hypocuprosis was observed in these animals and the disease was prevented by administration of copper.

Recently, ultrastructural alterations of neurons and the ultramicroscopic pathology of the cerebral lesions in swayback sheep have been reported (18, 19).

Until the recent report of Everson et al. (20), sheep were unique in that they were the only species reported to have nervous system abnormalities due to a deficiency of copper.[4] Everson et al. found that the young of guinea pigs fed a copper-deficient diet were ataxic and gross brain abnormalities were observed including agenesis of the cerebellum. Soft translucent areas occurred in the cerebral cortex of some guinea pigs.

Although copper deficiency in the rat has been rather extensively studied (21–23), a specific nervous disease has not been reported. During studies on the effect of a multipesticide mixture on reproduction of female rats receiving diets containing various concentrations of copper, signs of neurologic disturbances were observed in the litters of copper-deficient females. Although similar neural lesions were observed in copper-deficient animals receiving the pesticide mixture, the rats included in this report were not exposed to pesticides. These observations and a description of the histopathologic findings form the basis of this report.

MATERIALS AND METHODS

Experimental diets. The composition of the basal diet is given in table 1 and was adopted with certain modifications from the copper-deficient diet published by Mills and Murry (24). The modifications included the addition of starch and sucrose at the ratio found in contemporary American diets and the composition of the fat blend is comparable in fatty acid composition to diets consumed by Americans. The copper content of the basal diet was 0.9 ppm, and was determined by atomic absorption spectrophotometry by a private laboratory. Copper was added to the basal diet at a concentration of 8 ppm to provide the control diet.

TABLE 1
Composition of basal diet

	%
Corn starch	41.32
Sucrose	27.53
Casein	20.00
Fat blend [1]	5.00
Potassium phosphate, dibasic	1.94
Calcium carbonate	1.79
Sodium chloride	1.00
Calcium phosphate, dibasic	0.36
Vitamin [2] and mineral premixes [3]	1.06
Total	100.00

[1] Fat blend: (in grams per kilogram) edible beef tallow, 158.1; lard, 240.6; hydrogenated vegetable oil, 59.6; "Dexo" hydrogenated vegetable oil, 59.6; "Snowdrift" hydrogenated vegetable oil, 75.6; cottonseed oil, 75.6; soybean oil, 20.6; corn oil, 6.88; olive oil, 3.44; peanut oil, 3.44; milk fat, 264.9; "Blue Bonnet" margarine, 21.87; "Chiffon" margarine, 21.87; and "A & P" corn oil margarine, 21.87.

[2] Vitamin mixture as milligrams per kilogram of total diet: choline chloride, 715; inositol, 358; ascorbic acid, 72; calcium pantothenate, 29; *p*-aminobenzoic acid, 7; menadione, 18; thiamine, 21; riboflavin, 7; pyridoxine, 21; and niacin, 21; in μg per kilogram: biotin, 180; folic acid, 430; and vitamin B_{12}, 8.5; in units per kilogram: α-tocopheryl acetate, 75; vitamin A palmitate, 10,000; and vitamin D_2, 1000.

[3] Mineral mixture as milligrams per kilogram of total diet: copper, 0.9; zinc, 27; manganese, 75; magnesium, 734; iron, 300; and iodine, 36. Copper content was determined by atomic absorption spectrophotometry.

Experimental animals. Female rats of the Holtzman strain were purchased[5] when approximately 3 weeks of age and fed the experimental diets with 10 animals assigned per group. Rats fed the copper-deficient diet received glass-distilled water and all rats were housed in individual stainless steel cages. Feed and water were provided ad libitum. Females were mated when approximately 4 months of age with

[4] McGavin, M. D., P. D. Ranby and L. Tammemagi (Australian Vet. J., 38: 8, 1962) described demyelination of the spinal cord extending into the medulla oblongata and cerebellum in ataxic pigs with low levels of liver copper.

[5] Holtzman Company Animal Breeders, 421 Holtzman Road, Madison, Wis.

males fed commercial rat pellets.[6] Individual males were placed in breeding cages with three to four females and remained with the females for 5 days. Females were returned to their individual wire cages and were weighed on day 18 after placement with males and before transfer to maternity cages. Young were weaned on the same diets fed the dams.

Two breedings were completed and offspring which became moribund or exhibited severe neurologic signs were killed by ether overdosage. The age range of the 23 animals examined was from 30 to 50 days. These animals and those dying were necropsied; tissues were obtained for fixation in 10% buffered formalin. Fixed brain tissues were processed for paraffin sections, cut at 10 to 12 μ, and stained with hematoxylin and eosin. Selected sections were stained for myelin by the Kluver-Barrera method, for axons by the technique of Szatmari, for Nissl substance by Einarson's chrome alum gallocyanin, and Heidenhain's trichrome method was used for connective tissues. Frozen sections were postfixed in ammonium bromide formalin before staining for astrocytes by the gold chloride method of Cajal (25).

RESULTS

Clinical signs. Within 2 to 5 weeks, after the progeny of female rats were weaned to the copper-deficient diet, signs of neurological disturbance were observed in some animals. These signs were variable in intensity and were exacerbated by noise stimulation. Especially provoking was the sound of running water, either into the sink or when splashed onto the floor. Animals so stimulated would rush wildly about the cage. They would attempt to climb the cage walls and, when the cage was opened during one of the seizures, would leap onto the floor. Affected animals placed within a larger enclosure with high walls would run wildly about adjacent to the walls; also, they would cower in the corners, tremble, look wildly about and attempt to climb the walls. Many of the animals attempting to climb from the enclosure would cry out as if in pain. After a period of running, the paws were reddened apparently due to vascular congestion. The reactions during the seizures provoked by the auditory stimulation could be described as "running fits" and the response was one of extreme fright. When the stimulus was continued the animals would run to exhaustion and several succumbed within a few hours after prolonged stimulation. Within cages without noise stimulation, the signs were more catatonic in character; affected animals would remain without moving as if transfixed, and if limbs were placed in an awkward position, they were not readily returned to normal by the animal. A few animals displayed convulsive seizures; they would paddle with their forelegs and, rising on their rear legs, would fall over backwards. Some of the affected offspring were slightly ataxic and priapism was observed in many males.

Gross lesions. In reference to the nervous system, alterations observed at necropsy were minimal in most brains. Occasionally focal opaque areas (fig. 1), apparently necrotic, were found in various regions of the cerebral cortex including occipital and parietal, but the most severely affected was the frontal region which seemed atrophied and less vascular. The whitish areas were often slightly depressed below the surface of the surrounding normal cortex. Gross lesions were observed in other tissues, including the heart, and these will be reported separately.

Microscopic lesions. Major sites of predilection for neural lesions included various regions of the cerebral cortex and the corpus striatum of the telencephalon. Pathoanatomic alterations occurred within the frontal, parietal, occipital and cingulate regions of the cerebral cortex. Within the neocortex, involvement of tissues in the pathologic process was variable in extent and depth of penetration from the surface. Thus, the elements of the outer granular and adjacent layers were affected just beneath the molecular layer, or the focal lesions would involve the elements of the layer of polyhedral and large pyramidal cells without extension to the surface of the cortex (fig. 2). Often the involvement was in zones progressing from the surface of the cortex to the white matter (fig. 3). In some brains, a necrotic zone just beneath the molecular layer was followed by

[6] Purina Rat Chow, Ralston Purina Company, St. Louis, Mo.

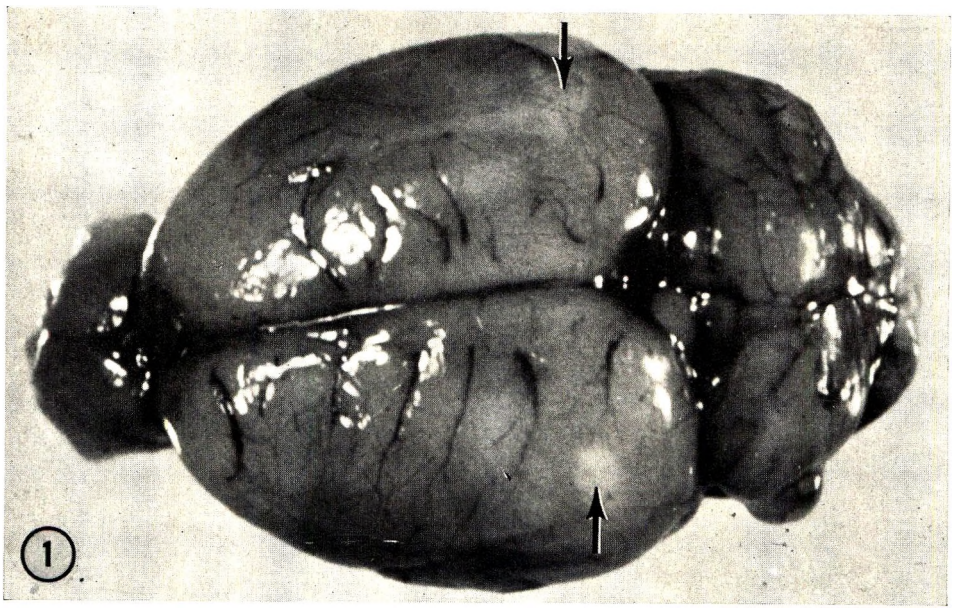


Fig. 1 Whitish foci (arrows) of apparent necrosis were noted at necropsy (fixed brain).

Fig. 2 Focal areas of edema and tissue rarefaction (arrows) are present in the cerebral cortex. Heidenhain stain. × 16.

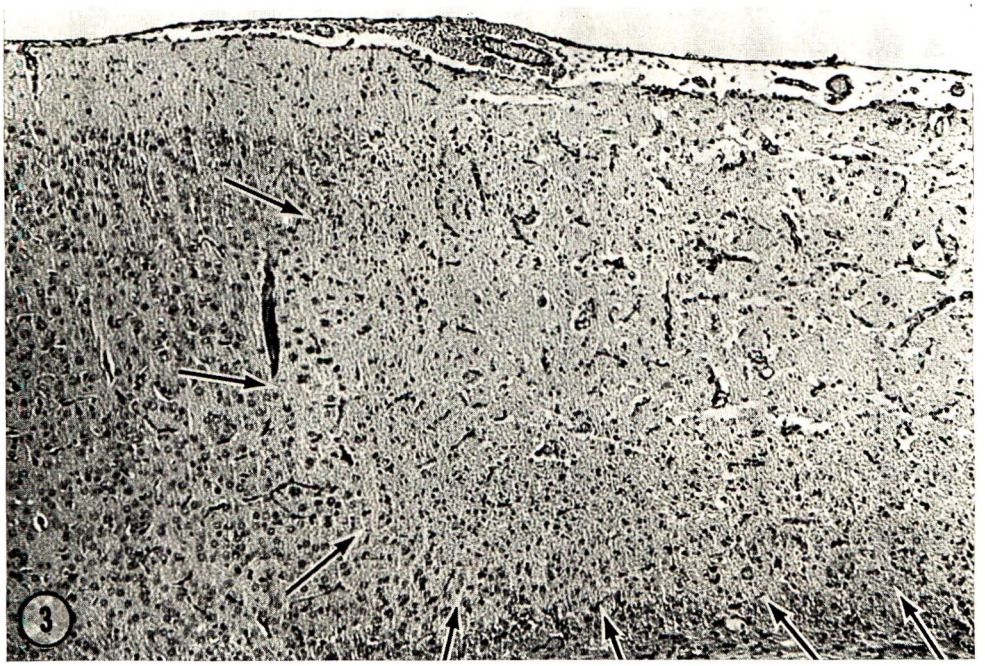


Fig. 3 Necrosis (outlined by arrows) often extended throughout the depth of the cerebral cortex. The necrotic areas were characterized by pale staining, loss of perikarya, and capillary proliferation. Hematoxylin and eosin. × 64.

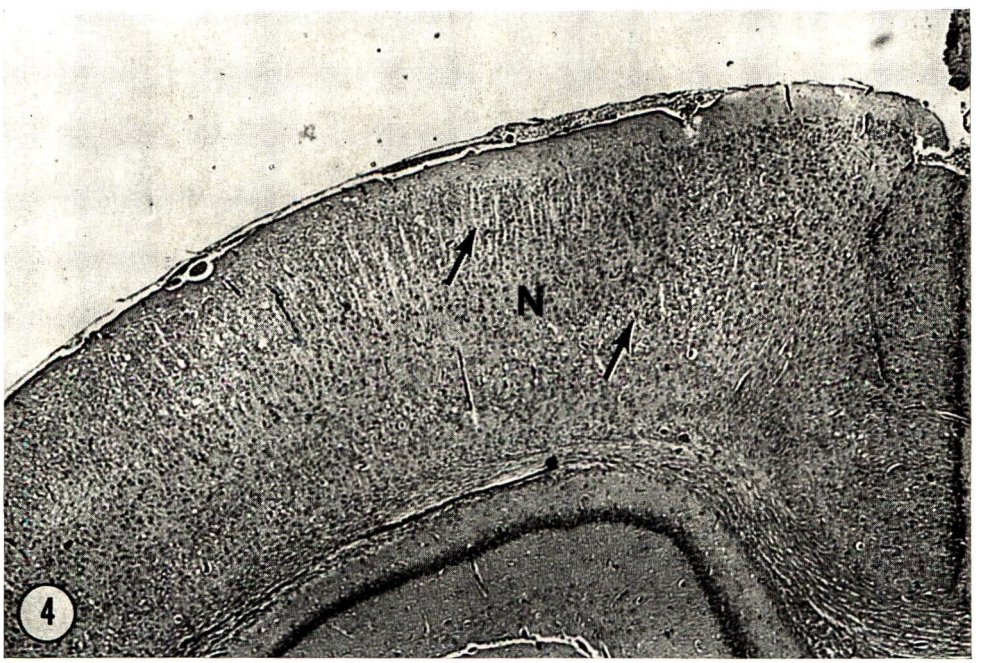


Fig. 4 In the cerebral cortex zones of necrosis and edema (arrows) were separated by tissue which appeared normal (N). Hematoxylin and eosin. × 25.

a zone of essentially normal perikarya bordering upon a deeper zone of necrosis and edema (fig. 4). Involvement of these major sites was not always concomitant; in some rats only the cortex contained lesions, in others only the region of the corpus striatum was affected, whereas in other rats lesions were observed in both cerebral cortex and corpus striatum. In most of the brains the lesions were bilateral and often symmetrical. Sections of the telencephalon which included the nucleus caudatus/putamen were studied most often, but the globus pallidus contained lesions as well. In the corpus striatum, as in the cerebral cortex, the extent of the lesions varied from small focal areas to extensive devastation of the neural tissue (figs. 5 and 6). Extensions of the lesions posteriorly to the diencephalon to involve portions of the anterior thalamic nuclei were noted in only a few rats (fig. 7).

Microscopic lesions were essentially those of coagulation necrosis and edema, but the extent of the necrosis varied widely from small microscopic foci to large areas which were obvious grossly. Affected areas presented rarefaction of tissues and were pale-staining. The lesions generally were sharply demarcated from normal cortex.

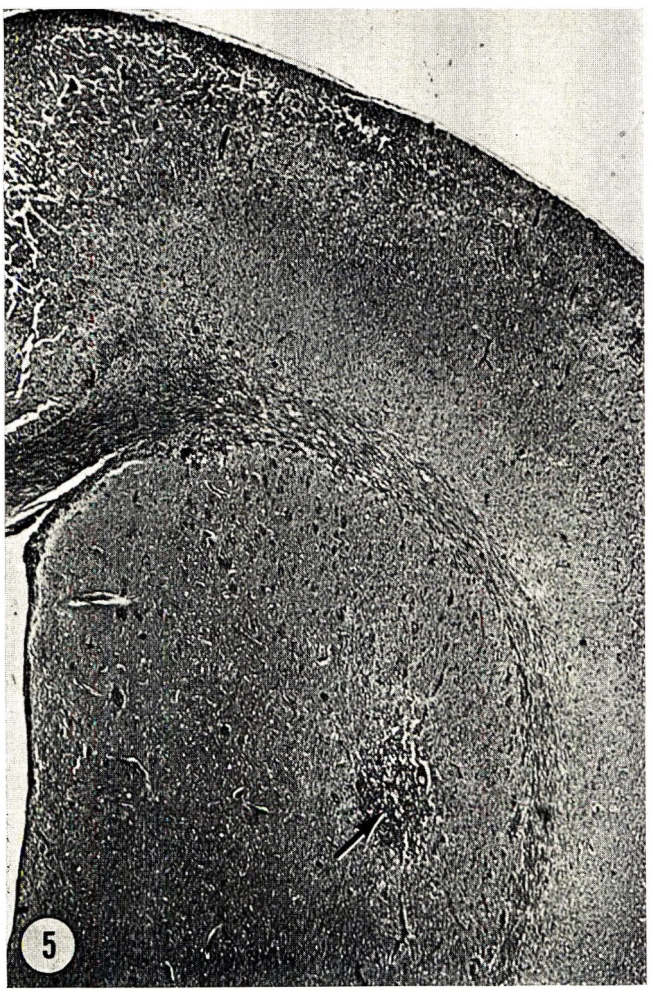


Fig. 5 Involvement of the corpus striatum in some brains consisted of small focal areas of tissue destruction (arrow). Heidenhain stain. × 20.

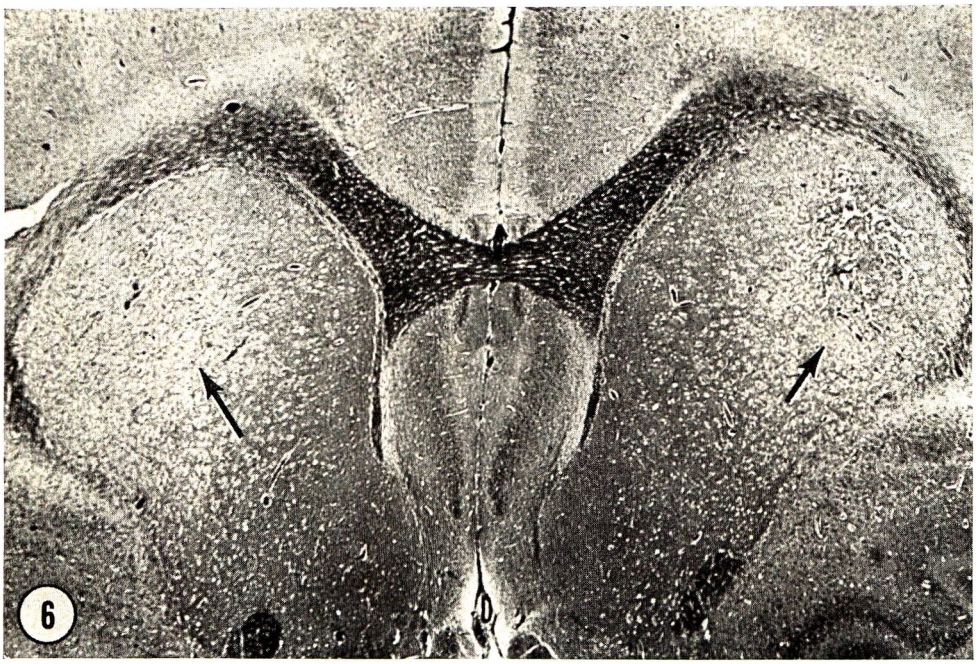


Fig. 6 In some of the brains there was extensive involvement of the tissues of the corpus striatum with tissue rarefaction and liquefactive necrosis (arrows). Kluver-Barrera stain. × 16.

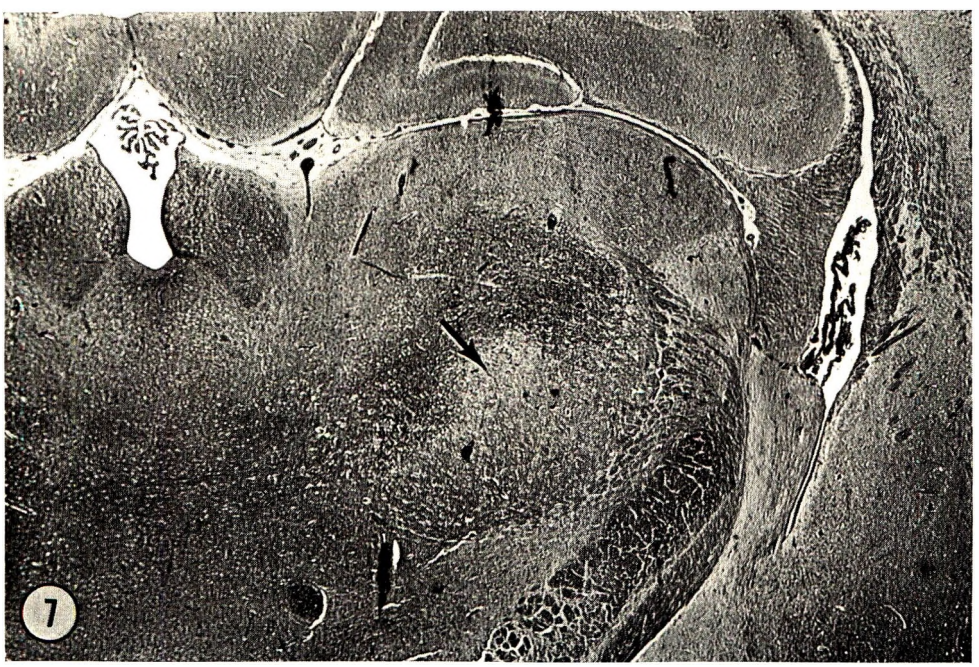


Fig. 7 Extension of the pathologic process with involvement of the thalamic region of the diencephalon was observed in a few brains (arrow). Heidenhain stain. × 16.

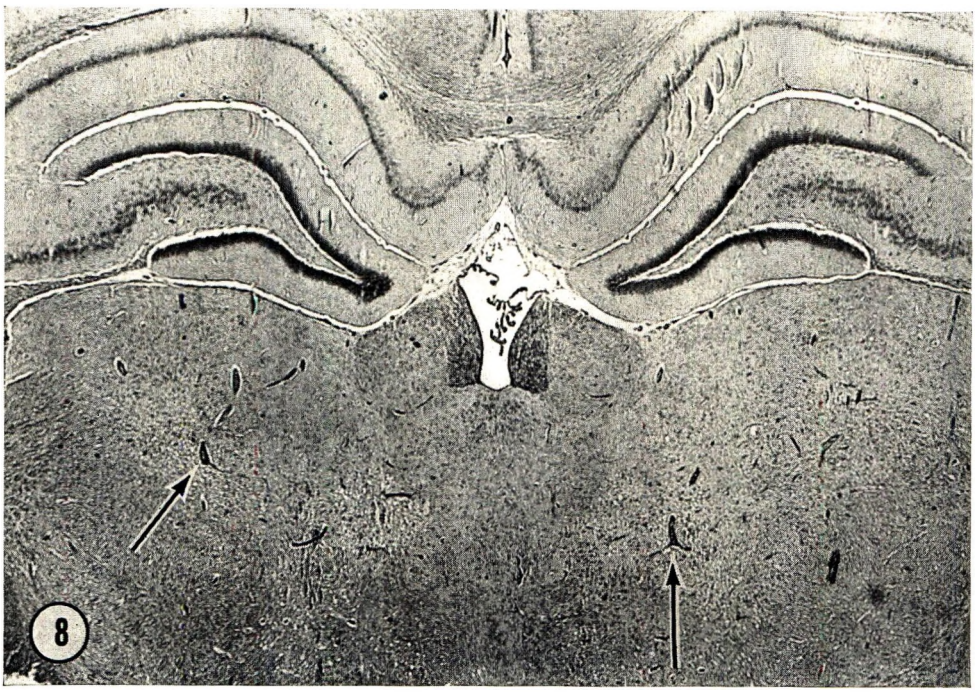


Fig. 8 Edema and tissue rarefaction were accompanied by proliferation of blood vessels, mainly capillaries (arrows). Hematoxylin and eosin. × 16.

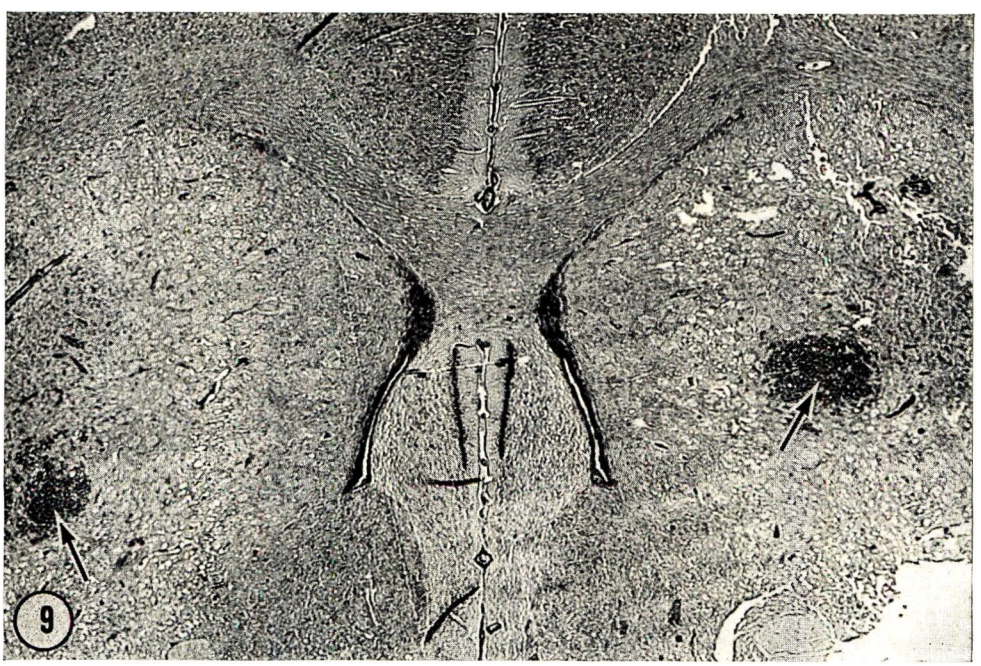


Fig. 9 Hemorrhage around vessels and within the parenchyma of the corpus striatum was observed in severely affected rats (arrows). Hematoxylin and eosin. × 20.

Nuclear pyknosis involved the perikarya of the cortex and the necrosis was accompanied by capillary proliferation so the affected areas appeared vascular (fig. 8). Some of the prominent vessels were metarterioles in which endothelial cell hypertrophy and hyperplasia of pericytes made the vessels stand out sharply within the rarefied regions of necrosis. Liquefaction involved some of the necrotic areas and portions were lost during tissue processing, although a minor residue of fat granule cells and tissue reticulum remained. Hemorrhage, both within the substance of the corpus striatum (fig. 9) and around the vessels, occurred in several brains but was not observed in the cerebral cortex. At the borders of liquefactive necrosis and hemorrhage, edema was severe, establishing a status spongiosus of the affected tissue.

In the pyriform cortex of a few rats necrosis of perikarya was evidenced by satellitosis and neurophagia. These reactive changes were not observed in the extensive areas of necrosis in the frontal and parietal cortex or the corpus striatum.

Although the necrotic areas were pale in myelin preparations demyelination was not severe or extensive. Stains for axis cylinders did not establish axonal degeneration as a feature of the pathologic process and destruction was limited to the necrotic areas in the corpus striatum. Other areas of the brain including the brain stem and cerebellum were without obvious lesions. No reactive astrogliosis was observed in sections stained by the method of Cajal. Secondary degenerative changes in the major lower motor neuron nuclei, as the red nucleus, apparently did not occur. No lesions were observed in the spinal cords examined.

Rats fed the control diet remained clinically normal during the period of observation and lesions did not occur in the brains of these animals.

DISCUSSION

The neural lesions observed in the copper-deficient rats of this study differ in many respects from the encephalopathy of hypocuprosis described in sheep (1–3, 10–12). Specifically, the syndrome did not parallel clinically or pathologically the description by Innes (2) of enzootic ataxia (swayback) as a diffuse symmetrical degeneration of the cerebral white matter did not occur. In the rat, changes were generally restricted to the cerebral cortex and the corpus striatum. The nerve fiber degeneration in the brain stem and spinal cord of swayback sheep considered essential by Barlow and co-workers (10–12) was not a feature of the neural disease in our rats.

Although differing in some respects, the nervous disease of the rats most resembled the lesions described in sheep by Roberts et al. (16, 17) in that cerebral edema and cortical necrosis were prominent findings in both species, but the microcavitation of the white matter and lesions of the spinal cord were not observed in rats. Everson et al. (20) did not describe the microscopic lesions in their guinea pigs, but the soft, translucent areas noted in the cerebral cortex suggest necrosis in these areas.

The pathogenesis of the edema and necrosis in the cerebral cortex and corpus striatum has not been established but would appear to be related to a biochemical disturbance of brain metabolism induced by inadequate amounts of copper. Copper has a specific role in brain metabolism because copper is an integral part of the cytochrome c oxidase molecule (26). Howell and Davison (27) demonstrated a significant reduction in cytochrome-oxidase activity in homogenates of frontal cortex, cerebellar peduncle and caudate nucleus prepared from brain tissues of lambs with swayback. A significantly lower cytochrome-oxidase activity in brain tissue from swayback lambs was confirmed by Mills and Williams (28). These data suggest that reduction of cytochrome-oxidase activity may be the biochemical lesion leading to the morphologic alterations of necrosis and edema. Tissue hypoxia due to a reduction in cytochrome-oxidase activity as the cause of the neural lesions is strengthened by numerous observations reviewed in Greenfield's book (29) that cyanide, carbon monoxide and other causes of hypoxia result in edema and necrosis of nervous tissue with sites of predilection including the cerebral cortex and corpus striatum. Cytochrome-oxidase levels were not determined in our material and the

specific site of the initial lesion is unknown. It may be located within the neurons or glial elements.

The neurologic disturbances observed in the test rats were probably due to the lesions of the cerebral cortex and corpus striatum. Studies with rats (30) and with cats (31) have established that behavioral disturbances follow destructive lesions of the corpus striatum. Zeman and Innes (32) have reviewed the fiber pathways involving the basal ganglia in the rat brain and have indicated that the fiber connections are in line with their functional significance as a relay center for motor expression of behavioral mechanisms.

LITERATURE CITED

1. Bennetts, H. W. 1932 Enzootic ataxia of lambs in Western Australia. Australian Vet. J., *8:* 137.
2. Innes, J. R. M. 1936 "Sway-back" — a demyelinating disease of lambs wtih affinities to Schilder's encephalitis in man. Vet. Rec., *48:* 1539.
3. Innes, J. R. M., and G. D. Shearer 1940 "Swayback" — a demyelinating disease of lambs with affinities to Schilder's encephalitis in man. J. Comp. Pathol. Therap., *53:* 1.
4. Bull, L. B., H. R. Marston, D. Murnane and E. W. L. Lines 1938 Ataxia in young lambs. Commonwealth Australia Council Sci. Ind. Res. Bull. no. 113, p. 23.
5. Bennetts, H. W., and A. B. Back 1942 Enzootic ataxia and copper deficiency of sheep in Western Australia. Commonwealth Australia Council Sci. Ind. Res. Bull. no. 147, p. 4.
6. McDonald, I. W. 1942 Enzootic ataxia of lambs in South Australia. Australian Vet. J., *18:* 165.
7. Behrens, H., and L. C. von Schulz 1959 Swayback (enzootische Ataxie) der Schalflammer. Deut. Tieraerztl. Wochensch., *66:* 502.
8. Schulz, L. C. von, and H. Behrens 1960 Beitrag zur Pathogenese der kupfer Mangelencephalopathie des Schaflammes (Swayback). Beitr. Pathol. Anat. Allg. Pathol., *122:* 282.
9. Spais, A. G., D. A. Palsson and L. van Bogaert 1961 Pathology of enzootic ataxia of lambs. Acta Neuropathol., *1:* 56.
10. Barlow, R. M. 1958 Recent advances in swayback. Proc. Roy. Soc. Med., *51:* 748.
11. Barlow, R. M., D. Purves, E. J. Butler and I. J. Macintyre 1960 Swayback in Southeast Scotland. I. Field aspects. J. Comp. Pathol., *70:* 396.
12. Barlow, R. M., D. Purves, E. J. Butler and I. J. Macintyre 1960 Swayback in Southeast Scotland. II. Clinical, pathological and biochemical aspects. J. Comp. Pathol., *70:* 411.
13. Barlow, R. M. 1963 Further observations on swayback. I. Transitional pathology. J. Comp. Pathol., *73:* 51.
14. Mills, C. F., and B. F. Fell 1960 Demyelination in lambs born of ewes maintained on high intakes of sulfate and molybdate. Nature, *185:* 20.
15. Butler, E. J., and R. M. Barlow 1963 Copper deficiency in relation to swayback in sheep. I. Effect of molybdate and sulphate supplements during pregnancy. J. Comp. Pathol., *73:* 208.
16. Roberts, H. E., B. M. Williams and A. Harvard 1966 Cerebral oedema in lambs associated with hypocuprosis, and its relationship to swayback. I. Field, clinical, gross anatomical and biochemical observations. J. Comp. Pathol., *76:* 279.
17. Roberts, H. E., B. M. Williams and A. Harvard 1966 Cerebral oedema in lambs associated with hypocuprosis, and its relationship in swayback. II. Histopathological findings. J. Comp. Pathol., *76:* 285.
18. Cancilla, P. A., and R. M. Barlow 1966 Structural changes of the central nervous system in swayback (enzootic ataxia) of lambs. II. Electron microscopy of the lower motor neuron. Acta Neuropathol., *6:* 251.
19. Cancilla, P. A., and R. M. Barlow 1966 Structural changes of central nervous system in swayback (enzootic ataxia) of lambs. III. Electron microscopy of the cerebral lesions. Acta Neuropathol., *6:* 260.
20. Everson, G. J., H. C. C. Tsai and T. I. Wang 1967 Copper deficiency in the guinea pig. J. Nutr., *93:* 533.
21. Gallagher, C. H., J. D. Judah and K. R. Rees 1956 The biochemistry of copper deficiency. I. Enzymological disturbances, blood chemistry and excretion of amino acids. Proc. Roy. Soc. (London) Ser. B, *145:* 134.
22. Gallagher, C. H., J. D. Judah and K. R. Rees 1956 The biochemistry of copper deficiency. II. Synthetic processes. Proc. Roy. Soc. (London) Ser. B, *145:* 195.
23. Gallagher, C. H. 1957 The pathology and biochemistry of copper deficiency. Australian Vet. J., *33:* 311.
24. Mills, C. F., and G. Murry 1960 The preparation of a semi-synthetic diet low in copper for copper-deficiency studies with the rat. J. Sci. Food Agr., *11:* 547.
25. Armed Forces Institute of Pathology 1960 Manual of Histologic and Special Staining Technics. McGraw-Hill Book Company, New York.
26. Wainio, W. W., C. V. Wende and N. F. Shimp 1959 Copper in cytochrome c oxidase. J. Biol. Chem., *234:* 2433.
27. Howell, J. McC., and A. N. Davison 1959 The copper content and cytochrome oxidase activity of tissues from normal and swayback lambs. Biochem. J., *72:* 365.
28. Mills, C. F., and R. B. Williams 1962 Copper concentration and cytochrome-oxidase

and ribonuclease activities in the brains of copper-deficient lambs. Biochem. J., *85:* 629.
29. Meyer, A. 1963 Intoxications. In: Greenfield's Neuropathology. Williams and Wilkins Company, Baltimore, Maryland, chapt. 4.
30. Harrison, J. M., and M. Lyon 1957 The role of the septal nuclei and components of the fornix in the behavior of the rat. J. Comp. Neurol., *108:* 121.
31. Mettler, F. A. 1948 Neuroanatomy. C. V. Mosby Company, St. Louis, Missouri.
32. Zeman, W., and J. R. M. Innes 1963 Craigie's Neuroanatomy of the Rat. Academic Press, New York.

Effects of Previous Calcium Intakes on Adaptation to Low and High Calcium Diets in Rats [1,2]

J. D. BENSON, R. S. EMERY AND J. W. THOMAS
Department of Dairy, Michigan State University, East Lansing, Michigan

ABSTRACT To study adaptation to low and high calcium diets, female weanling rats were fed for two periods in one of four ways: 1) a low calcium diet (0.2%) throughout both periods (LL); 2) a low calcium diet followed by a high calcium diet (0.9%) (LH); 3) a high calcium diet followed by a low calcium diet (HL); or 4) a high calcium diet throughout both periods (HH). Rats (LL and LH), accustomed to low intakes of calcium, retained a larger percentage of the dietary calcium than rats (HL and HH) accustomed to higher amounts of the element, regardless of whether the present calcium intake was low or high. Up to 133 days of age, the bones of the LL and LH groups contained less calcium and phosphorus per unit of bone weight than did the bones of the HL and HH groups. At 156 days of age (end of experiment), however, the bones of all groups contained equal concentrations of calcium and phosphorus. These data were interpreted to mean that body calcium stores control calcium retention. Parathyroid activity, as estimated by parathyroid gland weight and urinary phosphate values, was greater in the LL group than in the HL group. These data demonstrated that the adaptation to both high and low calcium diets was affected by previous dietary calcium levels and confirmed the idea that body stores of calcium control its retention.

The ability of animals to adapt to lowered or increased amounts of a nutrient by conserving or rejecting the nutrient in question is a general phenomenon in nutrition. Several studies (1–3) have shown that rats adapt to a decreased intake of calcium. Furthermore, these studies have demonstrated that rats previously fed a low calcium diet absorb a larger percentage of dietary calcium than unadapted rats when both groups are subsequently fed the same amounts of calcium. The mechanism whereby animals ingesting the same amounts of calcium absorb amounts inversely proportional to their previous calcium intakes remains a mystery. This research was initiated to study the effect of previous calcium diets on adaptation to both a low and a high calcium diet and to gain insight into the mechanism involved in such adaptation.

EXPERIMENTAL

The experiment was composed of a 60-day period (period 1) followed by a 75-day period (period 2). In addition, five rats per group were continued on the diets for 91 days. During period 1, 62 female weanling rats of the Sprague-Dawley strain (21 days of age) were fed a low calcium (0.22%) diet, whereas 48 similar rats were given a high calcium (0.94%) diet. At the conclusion of period 1, one-half the rats which had been fed the low calcium diet were switched to the high calcium diet for the duration of period 2. Similarly, one-half the rats fed the high calcium diet during period 1 were switched to the low calcium diet during period 2. The remaining rats continued with their original diets. According to these procedures, rats were fed in one of four ways as outlined in table 1.

The diets employed were similar to that of Henry and Kon (2) in all respects except for the calcium and phosphorus content (table 2). The phosphorus content of the high calcium diet was increased to avoid wide fluctuations in the calcium-to-phosphorus ratio between the two diets. The phosphorus content was adequate in both diets, whereas the calcium content was inadequate by NRC standards (4) in the low calcium diet.

Received for publication June 1, 1968.

[1] Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article no. 4387.

[2] Based on a portion of a thesis submitted by J. D. Benson to the Graduate School of Michigan State University in partial fulfillment of the requirements for the Master of Science degree.

TABLE 1

Experimental design

Treatment	No. of rats	Diet (level of Ca)	
		Period 1 60 days	Period 2 75 days
		%	%
Low-low (LL)	31	0.22	0.22
Low-high (LH)	31	0.22	0.94
High-low (HL)	24	0.94	0.22
High-high (HH)	24	0.94	0.94

TABLE 2

Composition of experimental diets

	Diet	
	Low calcium	High calcium
	%	%
Ground wheat	89.3	87.0
Dried skim milk	10.0	9.8
Vitamin and methionine premix [1,2]	0.1	0.1
Trace mineral salt [3]	0.5	0.5
Vitamin A, D and E mix [4]	0.1	0.1
$CaHPO_4$	—	2.5
Total	100.0	100.0
Ca	0.22	0.94
P	0.40	0.94

[1] The premix contained (in percent) choline chloride, 56.0; DL-methionine, 40.0; niacin, 2.6; vitamin B_{12}, 0.5; calcium pantothenate, 0.4; riboflavin, 0.3; and menadione, 0.2.

[2] NRC requirements for all B vitamins (4) were met by the contents of this mix and the calculated contents of wheat.

[3] Salt mix contained (in percent) NaCl, 96.5–98.6; manganese, 0.2; iron, 0.16; iodine, 0.141; copper, 0.033; cobalt, 0.010; and zinc, 0.005.

[4] Each gram of mix contained 33.0 IU vitamin A, 3.0 IU vitamin D, and 0.09 IU vitamin E.

Calcium and phosphorus balances were determined on 10 rats from each of the two treatments every 2 weeks for the duration of period 1. During period 2, balance trials were conducted on five rats from each of the four treatments at 2-week intervals. The same 20 rats were used in the balance trials throughout the entire experiment. Concomitantly, three rats from each treatment were killed every 2 weeks during period 2. Both tibiae and the parathyroid gland were removed and immediately frozen.

All rats were weighed every 10 days. Five rats from each treatment were bled every 2 weeks via heart puncture. The serum, feed, feces, bone and urine were analyzed for calcium and phosphorus. Feed, feces and bone were dried at 100° for 8 hours to determine a dry weight and then dry heated at 550° in a muffle oven until a clean white ash was obtained. The ash was dissolved in 5 ml of 6 N HCl and diluted to 100 ml with deionized water. This solution was analyzed for calcium and phosphorus. Calcium was analyzed by atomic absorption spectroscopy (5). The method of Gomori (6) was employed to determine phosphorus. Parathyroid glands were dissected free from the thyroid under a dissecting microscope and weighed on an electrobalance [3] to the nearest microgram.

TABLE 3

Body weight gains of rats fed varying or continuous levels of calcium

Treatment [1]	No. of rats	Body weight gain [2]	
		Period 1	Period 2
		g	g
LH	18	131.9 [a,3]	178.5 [a]
HH	13	127.8 [a,c]	177.0 [a]
HL	14	117.0 [b,c]	166.2 [a,b]
LL	18	120.0 [b,c]	160.6 [b]

[1] LH rats were fed low calcium diet for 60 days then high calcium diet for 75 days; HH rats, high calcium diet for the entire experiment (135 days); HL rats, high calcium diet for 60 days then low calcium diet for 75 days; and LL rats, low calcium diet for the entire experiment (135 days).

[2] Body weight at end of the period minus body weight at 25 days of age.

[3] Values in the same column with common superscripts are not significantly different by Duncan's multiple range test ($P \leqq 0.05$).

RESULTS AND DISCUSSION

Body weight. The LL group gained significantly less weight than the LH and HH groups for the duration of the experiment (table 3). The significant difference in weight gain between the LH and LL groups is only partially due to the diet during period 2, since a significant difference in weight gain occurred between these two groups at the concluson of period 1. Thus, a nonrandom selection of rats with respect to body weight accounted for much of the difference in weight gains between the LH and LL groups. The weight gains of the LL and HH groups, however, were not significantly different at the conclusion of period 1, but were different at the conclusion of the experiment. Thus, the continued feeding of a low calcium diet (LL) resulted in slightly but significantly lower weight gains when compared with diets (LH and HH) that contained ample amounts of calcium during the later stages of the growth

[3] Cahn Instrument Company, Paramount, California.

period. Sherman and Campbell (7) reported a greater weight gain in rats fed a 0.35%-calcium diet than in rats fed a 0.20%-calcium diet. Other investigators (8–10), however, have reported no differences in body weight gains among rats fed levels of calcium similar to those fed in these experiments.

Calcium retention. Rats fed the high calcium diet during period 1 retained significantly more calcium than the rats given the low calcium diet during that period (table 4). The rats offered the low calcium diet, however, utilized calcium much more efficiently than the rats fed the high calcium diet (table 4). Whereas rats given the low calcium diet ingested only 25% as much calcium as rats fed the high calcium diet, they retained 40 to 60% as much calcium as the rats fed the high calcium diet. These results agree with earlier reports (1, 2, 11) with rats that increased dietary calcium produced increased calcium retention but a decreased retention efficiency. This concept has also been described in dogs (12), dairy heifers (13) and humans (14).

During period 2 calcium retention was governed primarily by the level of calcium in the current diet and secondarily by the calcium level in the previous diet (table 4). Groups LH and HH, receiving the high calcium diet during period 2, retained approximately twice as much calcium as groups LL and HL, receiving the low calcium diet during that period, illustrating the marked effects of present dietary calcium level on calcium retention. As in period 1, groups LH and HH, receiving the high calcium diet, retained calcium much less efficiently than groups HL and LL, fed the low calcium diet during period 2 (table 4).

The effect of previous dietary calcium levels on current calcium retention can be demonstrated by comparing those groups which were fed the same calcium level during period 2 but which had been fed different levels of calcium during period 1. Thus, the comparisons of interest are LL versus HL and LH versus HH. The LL group retained more calcium than the HL group (114.4 versus 89.6 mg/5 days); likewise, the LH groups retained more calcium than the HH group (222.8 versus 197.6 mg/5 days). Previous studies showed that rats accustomed to a low calcium diet retained more calcium from a low calcium diet than rats accustomed to a high calcium diet (1, 2). Our data confirm this. Our data also demonstrated that rats previously fed a low calcium diet retained more calcium from a high calcium diet than rats accustomed to that diet. The differences in calcium retention between groups currently receiving the same level of calcium (LL versus HL and LH versus HH) were not as large as the differences

TABLE 4

Summary of calcium balance data

Treatment [1]	Calcium intake	Net calcium retention	Calcium retained
	mg calcium/rat/5 days		%
		Period 1 [2]	
Low	153.3	123.2 ± 4.2 [3]	76.7 ± 1.5 [4]
High	680.5	263.0 ± 13.0	34.4 ± 1.7
		Period 2 [5]	
LL	170.7	114.4 ± 4.8 [6]	66.8 ± 2.6 [7]
HL	166.5	89.6 ± 5.2	53.7 ± 2.8
LH	751.4	222.8 ± 22.9	33.9 ± 3.7
HH	801.3	197.6 ± 20.6	24.1 ± 1.8

[1] Treatment designations as in footnote 1, table 3.
[2] Values for this period are the means ± SE of three balance trials (10 rats each) conducted at 48, 62 and 76 days of age.
[3] High > low ($P < 0.01$).
[4] Low > high ($P < 0.01$).
[5] Values for this period are the means ± SE of four balance trials conducted at 90, 104, 118 and 132 days of age.
[6] LH and HH > LL and HL ($P < 0.01$); LL > HL ($P < 0.05$).
[7] LL and HL > LH and HH ($P < 0.01$ by orthogonal contrasts); LL > HL ($P < 0.05$ by orthogonal contrasts); and LH > HH ($P < 0.05$ by orthogonal contrasts).

between groups currently receiving different levels of calcium (HL and LL versus LH and HH). Thus, calcium retention was affected more by the current dietary calcium level than by the previous level. When the calcium retentions were summed for period 2, the total calcium retention of the LL group exceeded that of the HL group by approximately 300 mg. Likewise, the LH group retained 300 mg more calcium than did the HH group during period 2.

The greater calcium retention of the LL group compared with the HL group was not due to a difference in calcium intake, as in period 1, but was a factor of increased percentage retention (table 4). An increased percentage retention could be interpreted as an adaptive mechanism to an altered calcium intake and reflects the ability of an animal to retain calcium in inverse proportion to its previous calcium intake. Most of the calcium was excreted via the feces (table 5), indicating that the adaptive mechanism occurred at the intestinal level, most likely by changes in intestinal absorption. These data do not exclude the possibility that fecal endogenous calcium, which increases with increasing dietary calcium (9, 15), was increasing in the rats fed the high calcium diets. Increases in fecal endogenous calcium in response to greater calcium intakes, however, may be due to a decreased reabsorption rate of the endogenously secreted calcium and not due to an increased secretion rate per se.

As seen in table 5 and as suggested by Hansard and Crowder (16), fecal calcium values of rats fed the same level of calcium (LL versus HL and LH versus HH) were indicative of the calcium status of the animal.

TABLE 5
Calcium excretion of rats fed continuous or varying amounts of calcium

Age	Treatment [1]	Urine calcium [2]	Fecal calcium [2]
days		*mg/rat/5 days*	
90	LL	8.4 ± 0.5	14.5 ± 7.4
104	LL	12.0 ± 1.7	37.8 ± 11.1
118	LL	13.3 ± 3.2	53.1 ± 12.2
132	LL	12.4 ± 2.0	66.9 ± 4.3
90	HL	5.3 ± 2.9	53.2 ± 5.2
104	HL	7.4 ± 1.4	60.6 ± 8.8
118	HL	10.1 ± 0.7	68.0 ± 10.0
132	HL	9.6 ± 2.3	93.7 ± 8.6
90	LH	16.2 ± 3.5	372.7 ± 12.0
104	LH	19.6 ± 3.8	474.9 ± 33.8
118	LH	26.0 ± 2.2	531.4 ± 32.0
132	LH	14.5 ± 3.8	631.7 ± 45.3
90	HH	19.7 ± 4.5	517.8 ± 11.0
104	HH	16.7 ± 4.3	535.6 ± 17.8
118	HH	24.1 ± 5.7	630.4 ± 25.6
123	HH	20.8 ± 4.6	649.7 ± 36.7

[1] Treatment designations as in footnote 1, table 3.
[2] Values are means ± SE for five rats at each age.

TABLE 6
Concentration of calcium and phosphorus in tibiae of rats fed continuous or varying amounts of calcium

Treatment [1]	Age	
	105–133 days	156 days
	% of dry bone	*% of dry bone*
Calcium		
LL	[a] 21.4 ± 0.5(9) [2]	[a] 23.4 ± 0.6(11)
LH	[a] 21.2 ± 0.3(8)	[a] 23.1 ± 0.2(11)
HL	[b] 23.5 ± 0.3(9)	[a] 22.9 ± 1.0(7)
HH	[b] 23.4 ± 0.4(9)	[a] 23.2 ± 1.0(3)
Phosphorus		
LL	[a] 10.3 ± 0.1(9)	[a] 10.5 ± 0.1(10)
LH	[a] 10.3 ± 0.2(8)	[a] 10.7 ± 0.1(11)
HL	[b] 11.1 ± 0.2(8)	[a] 10.9 ± 0.2(7)
HH	[b] 11.0 ± 0.4(9)	[a] 11.2 ± 0.4(3)

[1] Treatment designations as in footnote 1, table 3.
[2] Mean ± SE for number of rats indicated in parentheses. Values in the same column with common superscripts are not significantly different by Duncan's multiple range test ($P \leqq 0.05$).

Bone. At 105 to 133 days of age, the tibiae of the LL and LH groups contained less calcium per unit dry bone weight than tibiae of the HL and HH groups (table 6). At the conclusion of the experiment, at 156 days, however, the calcium content of the tibiae did not differ among treatments. This result indicated that the concentration of bone calcium in the rats previously fed a low calcium diet was continuing to increase during period 2, whereas the bone calcium concentration of the rats fed the high calcium diet during period 1 was maximal at 105 to 133 days of age. In a similar study, Henry and Kon (2) demonstrated that calcification was dependent on the calcium content of the diet during the growth period, but at 1 year of age calcification was similar regardless of the dietary calcium level.

The smaller concentration of calcium in the bones of the LL and LH groups than

in the HL and HH groups at 105–133 days of age may be due not to a diminished calcium accretion rate of the former groups but to an accelerated bone resorption rate. The recent observations of Cohn et al. (15) would support this contention. To explain the equal concentrations of bone calcium in all groups at 156 days of age, one must assume that either the bone accretion rates of the LL and LH groups were greater than those of the HL and HH groups during this period (133 to 156 days of age) or that the bone resorption rates of the LL and LH groups were significantly less than those of the HL and HH groups. Our data do not distinguish between these two possibilities.

A similar situation existed when bone phosphorus was considered. The bones of the HL and HH groups contained significantly more phosphorus than did those of the LH and LL groups between the ages of 105 and 133 days (table 6). Like calcium, however, the phosphorus concentrations of the bones were not different among groups at the conclusion of the experiment. Although the concentrations of bone phosphorus were not significantly different among groups at 156 days of age, the phosphorus concentration of the LL and LH groups did not increase between 105 to 133 days and 156 days to the same extent as did the calcium concentration during that time (table 6). Because the phosphorus content of the low calcium diet presumably was adequate and since phosphorus is deposited in bone as calcium phosphates, the reason for a bone unsaturated with respect to phosphorus between 105 and 133 days of age was most likely a calcium rather than a phosphorus deficiency at the site of calcification. Thus, rats fed a low calcium diet can adapt to it so that at 156 days of age their skeletons contain the same concentrations of calcium and phosphorus as rats which have received more liberal amounts of the element.

Relationship between bone calcium and calcium retention. Nicolaysen (3) suggested that calcium absorption is regulated by a humoral factor from the osteoblasts which he termed "endogenous factor." This factor would be secreted during periods when calcium is being rapidly deposited in the bone. According to Nicolaysen's hypothesis, the endogenous factor acts on the intestine to increase calcium absorption. This proposal is an adequate explanation for the observations that the LL group retained more calcium during period 2 than the HL group and that the LH group retained more calcium than the HH group during period 2. As already noted, the concentration of calcium in the bones of both the HL and HH groups had attained maximal values shortly after the conclusion of period 1, whereas the bone calcium of the LL and LH groups did not reach maximal values until 133 to 156 days of age (table 6). Thus, the increased calcium retention of the groups fed the low calcium diet during period 1 (LL versus HL and LH versus HH) could be explained by the theory that bone stores are partially controlling calcium absorption. The manner in which the absorption of calcium could be increased at the intestinal level is open to question. One possibility is that some stimulation resulting from the low calcium stores in the bone could induce the synthesis of a carrier protein which would serve to transfer more calcium across the intestine. Wasserman and Taylor (17) showed that vitamin D_3 induced such a protein when given orally to chicks. Furthermore, calcium absorption did not increase in response to a previous low calcium diet if the animal was deficient in vitamin D (18). Perhaps a humoral factor from bone acting in combination with vitamin D could act to control calcium absorption through the intestine.

Calcium retention decreased linearly with age in all groups (fig. 1), in agreement with earlier studies (2, 16, 19). Since age was positively correlated with body stores of calcium, this factor and not age itself was probably responsible for the reduced calcium retention at the later ages. On the basis of these data and those from the studies mentioned, the conclusion that submaximal body stores of calcium were responsible for the increase retention and percentage utilization exhibited by the LL group seems unavoidable. Conversely, the maximal calcium stores of the HH and HL groups would serve to reduce calcium absorption and thus prevent further calcium deposition. Thus, in a sense, the system is

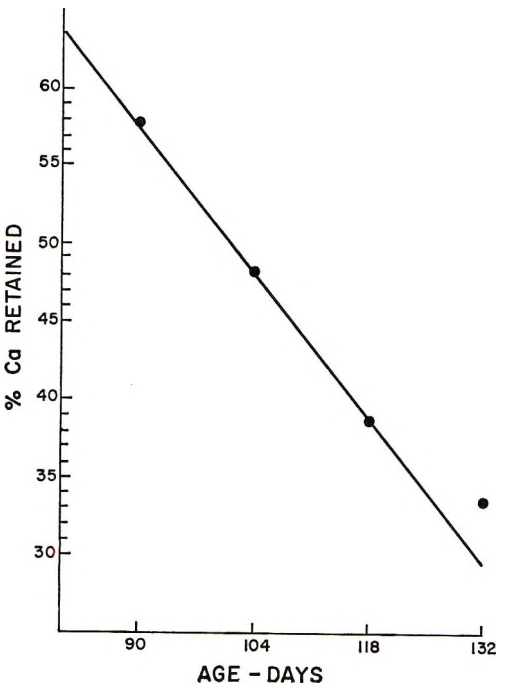


Fig. 1 Effect of age on percentage calcium retained. Each point represents mean of 20 rats (from each of the treatments). Response was significantly linear ($P \leq 0.05$).

one of end-product inhibition, where bone calcium acts as the end product and controls the initial step of calcium entry into the body proper, that is, absorption through the intestine. Ali and Evans (20) demonstrated a decrease in pH of the gastrointestinal tract when a low calcium (0.19%) diet was fed. They suggested that this effect may explain the increased efficiency of calcium absorption when low calcium diets are fed. A decrease in pH would serve to increase the concentration of ultrafiltrable, soluble and nonbound calcium (21). Although changes in pH may explain differences in calcium absorption between groups fed widely different levels of calcium, they would not explain differences in calcium absorption between groups fed the same level of calcium, that is, LL versus HL and LH versus HH.

Serum calcium. The analyses of serum calcium revealed two noteworthy differences: 1) Serum calcium increased significantly with age; 2) the LL and LH groups maintained higher serum calcium values throughout the experimental period than did the HL and HH groups (table 7). The level of serum calcium is generally considered to be independent of age, although few controlled studies have been reported to test this hypothesis. The means by which a high calcium intake depresses serum calcium levels (group HL) up to 156 days of age, or 75 days after the high calcium diet was last fed, remain unknown. Thus, analysis of serum calcium provided no insight into the mechanism of calcium adaptation but did raise several perplexing and as yet unexplainable questions regarding the effects of these treatments and age on serum calcium.

Parathyroid gland. The role of parathormone, a hormone secreted by the parathyroid glands, in maintaining serum calcium levels is well established. Whether this hormone plays a dominant role in increasing calcium retention during long periods of calcium deprivation is not clear. Several studies (22–25) have indicated that low intakes of calcium stimulated parathy-

TABLE 7

Serum calcium levels of rats fed continuous or varying amounts of calcium

Age	Treatment [1]			
	LL	LH	HL	HH
days	*mg calcium/100 ml*		*mg calcium/100 ml*	
90	6.8±0.4(4) [2,3]	6.3±0.5(4)	5.9±0.1(5)	6.4±0.1(5)
104	7.3±0.2(5)	7.9±0.3(5)	7.6±0.1(5)	7.8±0.4(5)
118	8.4±0.2(5)	8.3±0.4(5)	7.6±0.2(4)	7.8±0.1(5)
132	8.2±0.2(5)	8.5±0.3(4)	7.6±0.4(5)	7.4±0.8(5)
156	9.0±0.4(12)	9.1±0.7(10)	7.7±1.3(7)	6.2±1.1(3)
247	10.0±0.1(5)	9.9±0.1(6)	9.4±0.3(5)	9.8±0.3(6)

[1] Treatment designations as in footnote 1, table 3.
[2] Serum calcium values are significantly different for different ages and for different treatments ($P \leq 0.05$, by unweighted means analysis by variance).
[3] Values are the mean ± SE. The number in parentheses represents the number of rats per treatment combination.

roid activity. Nordin (26), however, concluded that low calcium diets did not affect parathyroid activity and that a bone tissue fluid equilibrium was responsible for correcting small changes in serum calcium.

The size and weight of the parathyroid gland has provided an estimate of the activity of the gland (24). In this experiment the parathyroids of the LL and HL groups were heavier than those of the LH and HH groups during period 2 (table 8). Also the parathyroids of the LL group were significantly heavier than those of the HL group, indicating that the previous calcium intake affected the weight and presumably the activity of the parathyroid glands of these rats. The greater parathyroid size of the LL group when contrasted to the HL group can most likely be attributed to a longer exposure to the low calcium diet. Higher urinary phosphate values are considered by some investigators (26) to reflect parathyroid secretion. In this respect the average daily urinary phosphate values for the LL and HL groups during period 2 were 24.8 and 20.7 mg, respectively. In contrast, the parathyroid weights of the LH and HH groups were not significantly different (table 8). The average daily urinary phosphate values for the LH and HH groups were 36.8 and 34.0 mg, respectively. Thus, using parathyroid weight and urinary phosphate values as criteria for parathyroid activity, it appears that the parathyroids of the LL group were more active than those of the HL group, whereas there was no significant difference in parathyroid activity between the LH and HH groups. Proof of parathyroid involvement in the adaptive mechanism necessitates a more sensitive assay of parathormone such as that described by Berson et al. (27). Evidence, however, that the parathyroid was not involved in the adaptive response has been reported by Gran (28), who demonstrated that parathyroidectomized rats were as capable of responding to a previously fed low calcium diet as intact rats.

Thyrocalcitonin, which inhibits bone resorption (29) and thus functions to reduce serum calcium, is a conceivable mediator of the adaptation response. Its failure to affect calcium absorption (30, 31), however, and Bronner's [4] observations that the thyrocalcitonin contents of thyroid glands from rats fed various levels of calcium did not differ, make it an unlikely candidate as a mediator of the adaptive response in this experiment.

TABLE 8

Parathyroid gland weights of rats fed continuous or varying amounts of calcium

Treatment [1]	No. of rats	Gland weight [2]
		μg/g body wt
LL	21	68.7 [a]
LH	16	53.0 [c]
HL	15	62.7 [b]
HH	12	57.0 [b,c]

[1] Treatment designations as in footnote 1, table 3.
[2] Values with the same superscript are not significantly different by Duncan's multiple range test ($P \leq 0.05$).

LITERATURE CITED

1. Rottensted, K. V. 1938 The effect of body stores on the efficiency of Ca utilization. Biochem. J., 32: 1285.
2. Henry, K., and S. K. Kon 1953 The relationship between calcium retention and body stores of calcium in the rat; effect of age and vitamin D. Brit. J. Nutr., 7: 147.
3. Nicolaysen, R. 1943 The absorption of Ca as a function of the body saturation with Ca. Acta Physiol. Scand., 5: 200.
4. National Research Council, Committee on Animal Nutrition 1962 Nutrient Requirements of Laboratory Animals, publ. 990. National Academy of Sciences — National Research Council, Washington, D. C.
5. Willis, J. B. 1960 Determination of Ca in blood serum by atomic absorption spectroscopy. Nature, *186:* 249.
6. Gomori, G. 1953 Standard Methods of Clinical Chemistry, vol. 1. Academic Press, New York.
7. Sherman, H. C., and H. L. Campbell 1935 Effects of increasing the Ca content of a diet in which Ca is one of the limiting factors. J. Nutr., *10:* 363.
8. Copp, D. H., and A. P. Suiker 1962 Study of calcium kinetics in calcium and phosphorus deficient rats with the aid of radio-calcium. In: Symposium on Radioisotopes and Bone, eds., F. C. McLean and P. Lacroix. Blackwell Scientific Publications, Oxford, England.
9. Hansard, S. L., and M. P. Plumlee 1954 Effects of dietary calcium and phosphorus levels upon the physiological behavior of calcium and phosphorus in the rat. J. Nutr., 54: 17.

[4] Bonner, F., S. Hurwitz, P. Sammon and B. Shah 1967 Thyrocalcitonin content of thyroid glands from rats on various calcium intakes. Federation Proc., 26: 367 (abstract).

10. Evans, J., and R. Ali 1967 Calcium utilization and feed efficiency in the growing rat as affected by dietary calcium, buffering capacity, lactose and EDTA. J. Nutr., *92:* 417.
11. Fairbanks, B., and H. Mitchell 1936 The relation between calcium retention and the store of calcium in the body with particular reference to the determination of calcium requirements. J. Nutr., *11:* 551.
12. Gershoff, S., M. Legg and D. Hegsted 1958 Adaption to different calcium intake in dogs. J. Nutr., *64:* 303.
13. Lindsey, J., J. Archibald and P. Nelson 1931 The calcium requirements of dairy heifers. J. Agr. Res., *42:* 883.
14. Malm, O. 1963 Adaptations to alterations in calcium intake. In: The Transfer of Ca and Sr Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York.
15. Cohn, S., T. Teree and E. Gusmano 1968 Effect of varying calcium intake on the parameters of calcium metabolism in the rat. J. Nutr., *94:* 261.
16. Hansard, S. L., and H. M. Crowder 1957 The physiological behavior of calcium in the rat. J. Nutr., *62:* 325.
17. Wasserman, R. H., and A. N. Taylor 1966 A vitamin D_3-induced Ca binding protein in chick intestinal homogenates. Science, *152:* 791.
18. Nicolaysen, R., N. Eeg-Larsen and O. Malm 1953 Physiology of Ca metabolism. Physiol. Rev., *33:* 424.
19. Taylor, D. M., P. H. Bligh and M. Duggan 1962 The absorption of Ca, Sr, Ba and radium from the GI tract of the rat. Biochem. J., *83:* 25.
20. Ali, R., and J. Evans 1967 Effect of dietary calcium, buffering capacity, lactose and EDTA on pH and calcium absorption from gastrointestinal segments of the growing rat. J. Nutr., *93:* 273.
21. Storry, J. 1961 Studies on calcium and magnesium in the alimentary tract of sheep. 2. The effect of reducing the acidity of the abomasal digesta in vitro on the distribution of calcium and magnesium. J. Agr. Sci., *57:* 103.
22. Luce, E. M. 1923 The size of the parathyroid of rats and the effect of a diet deficient in Ca. J. Pathol. Bacteriol., *26:* 200.
23. Ham, A., W. Littner, T. Drake, E. Robertson and F. Tisdall 1940 Physiological hypertrophy of the parathyroids. Its cause and relation to rickets. Amer. J. Pathol., *16:* 277.
24. Harrison, M., and R. Fraser 1960 The parathyroid gland and calcium deficiency in the rat. J. Endocrinol., *21:* 207.
25. Au, W., and L. G. Raisz 1965 Effect of vitamin D and dietary calcium on parathyroid activity. Amer. J. Physiol., *209:* 637.
26. Nordin, B. E. C. 1960 Osteoporosis and calcium deficiency. In: Bone As a Tissue, eds., K. Rodahl, J. I. Nicolson and E. M. Brown, Jr. McGraw-Hill Book Company, New York.
27. Berson, S. A., R. S. Yalow, D. Aurbach and J. T. Potts 1963 Immunoassay of bovine and human parathyroid hormone. Proc. Nat. Acad. Sci., *49:* 613.
28. Gran, F. C. 1960 Studies on Ca and Sr^{90} metabolism in rats. I. Studies on digestive juices. II. Long term experiments of Sr^{90} metabolism. III. The excretion and resorption of Ca in the skeleton. Acta Physiol. Scand., *48* (suppl.): 1.
29. Freedman, J., and L. Raisz 1965 Thyrocalcitonin: Inhibitor of bone resorption in tissue culture. Science, *150:* 1465.
30. Krawitt, E. 1967 Effect of thyrocalcitonin on duodenal calcium transport. Proc. Soc. Exp. Biol. Med., *125:* 1084.
31. Bronner, F., P. Sammon, R. Stacey and B. Shah 1967 Role of thyrocalcitonin in the regulation of the blood calcium level. Biochem. Med., *1:* 261.

Serum Proteins in Guinea Pig Scurvy [1]

N. P. TORRE [2] AND F. A. GREEN [2]
Buffalo General Hospital and Department of Medicine, State University of New York at Buffalo, Buffalo, New York

ABSTRACT Reported changes in gamma globulin levels in guinea pig scurvy have differed, in part reflecting the type of controls used. The present study, undertaken to investigate serum protein changes in guinea pig scurvy, and to correlate these with the possible development of amyloidosis, showed no differences in gamma globulin levels between the experimental group and the controls. A constant finding was a decrease in serum albumin and an increase in alpha-1 globulin. Amyloidosis did not develop. Immunological procedures to study the development of scurvy-specific serum antigenic determinants were negative.

Guinea pig scurvy is of considerable interest because ascorbic acid depletion is the only instance recorded in the literature in which the omission of a single chemical substance from the diet has resulted in what was first termed an eosinophilic infiltrate (1) and later, identified by staining techniques, as amyloid (2, 3). In conjunction with an attempt to study this further, the serum protein alterations in guinea pig scurvy were characterized. Data from other such investigations have been divided as to whether serum gamma globulin changes occurred (4–6). One of the reasons for these discordant results may be that previous studies have compared individual or pooled scorbutic guinea pig sera with "normals," without note being made of any attempt to match controls for weight or age. The present studies indicate that these variables are of importance, especially with regard to serum gamma globulin. Two types of control sera are included: first, sera obtained from bleeding the experimental group prior to scorbutogenesis, and second, sera from another group matched for weight with the experimental group, fed the same scorbutogenic diet but supplemented with injections of vitamin C.

METHODS

Random bred, weanling, male and female guinea pigs, 100 to 200 g in weight, were divided into two groups; they were matched for weight and bled from the retro-ocular veins. The experimental group was fed unsupplemented commercial rabbit ration,[3] containing all other vitamins, and the control group was fed the same diet supplemented with 4 mg ascorbic acid, injected intraperitoneally 5 days/week. All animals were weighed three times weekly. The criteria for the development of scurvy were: severe progressive weight loss, stumbling gait and hemorrhages into the hind limbs. No such manifestations were noted in any of the control group. The onset of scorbutic symptoms varied from 3 to 6 weeks. When the signs appeared the animals were bled a second time.

At the end of the first 2-month period the average weight of the animals fed commercial rabbit ration supplemented with 4 mg vitamin C/day was 2.5 times the initial weight. In contrast, the average weight of those surviving in the experimental group was 15% less than the initial

Received for publication June 10, 1968.

[1] Supported in part by Public Health Service Research Grant no. AM-05581 and United Health Foundation of Western New York Research Training Grant no. F-64-BGH-1.

[2] Present address: Veterans Administration Hospital, 3495 Bailey Avenue, Buffalo, New York.

[3] Purina Rabbit Checkers, Ralston Purina Company, St. Louis. Chemical composition is (in percent) protein, 15.18, in which all amino acids (as percentage of total ration) are arginine, 0.80; glycine, 0.41; lysine, 0.62; methionine, 0.27; tryptophan, 0.18; cystine, 0.25; histidine, 0.30; leucine, 1.17; isoleucine, 0.82; phenylalanine, 0.71; threonine, 0.57; and valine, 0.69; fat, 2.46; fiber, 15.25; ash, 7.43; and nitrogen-free extract, 46.86. The mineral constituents are (in percent) calcium, 1.15; phosphorus, 0.57; potassium, 1.21; magnesium, 0.26; sodium, 0.21; chlorine, 0.43; and (in ppm) iron, 288.55; zinc, 24.59; manganese, 41.67; copper, 11.22; cobalt, 0.23; and iodine, 0.55. The vitamin mixture is vitamin A, 0.50 IU/g; vitamin D, 2.25 IU/g; alpha-tocopherol, 32.47 IU/lb.; vitamin B_{12}, 0.30 μg/lb.; and (in ppm) carotene, 23.95; thiamine, 4.48; riboflavin, 7.94; niacin, 38.18; pantothenic acid, 16.18; choline, 927.00; folic acid, 2.93; pyridoxine, 2.81; and biotin, 0.18.

weight at the end of 2 months. No animal fed the unsupplemented diet was able to hold its initial weight at the end of the 2-month period and many did not survive.

Another group of animals having a higher average initial weight than the previous group was fed normal unsupplemented guinea pig ration. At the end of 2 months the average weight was 1.76 times the initial weight. A comparable group of animals fed rabbit ration supplemented with 4 mg vitamin C had essentially the same weight increment. This indicates that the animals fed rabbit ration plus 4 mg vitamin C daily were normal by growth criteria.

In addition, a group of animals was allowed to develop disease and half was then treated wtih vitamin C. The treated group recovered and began to grow normally but all animals in the untreated group died.

Microzone serum protein electrophoresis was carried out on cellulose acetate strips. Total proteins were measured by the biuret method (7).

To study possible new serum protein antigens, pooled normal guinea pig serum was injected intracutaneously into the abdominal wall of three normal rabbits using multiple sites. After immunization for 5 weeks the rabbit whose serum provided the most satisfactory levels of precipitating antibodies against guinea pig serum (as judged by immunoelectrophoresis) was given a further course of injections with pooled scorbutic guinea pig sera. The rabbit antiscorbutic guinea pig serum was completely absorbed with normal guinea pig serum, such that no lines were visible by double diffusion in agar. The absorbed antiscorbutic guinea pig serum was then reacted in double diffusion plates with the individual scorbutic guinea pig sera.

RESULTS AND DISCUSSION

The means and standard errors fail to take into account changes occurring in *individual* guinea pigs. Figures 1, 2 and 3 indicate the changes in individual animals; the means of two or more determinations were used. During induction of the scorbutic state the albumin fell and the alpha-1 globulin rose in the scorbutic group but were unchanged in the group which was supplemented with vitamin C (control group). The changes in albumin (reduction) and alpha-1 (increase) are dramatic in each individual animal, a uniformity not ascertainable from the means and standard errors. In addition, as a group these changes were statistically significant (*P* less than 0.01 or better). The gamma globulin changes, however, were more variable. The serum gamma globulin tended to rise in both groups indistinguishably. The overall rise in gamma globulin in both groups appears to be age-related.

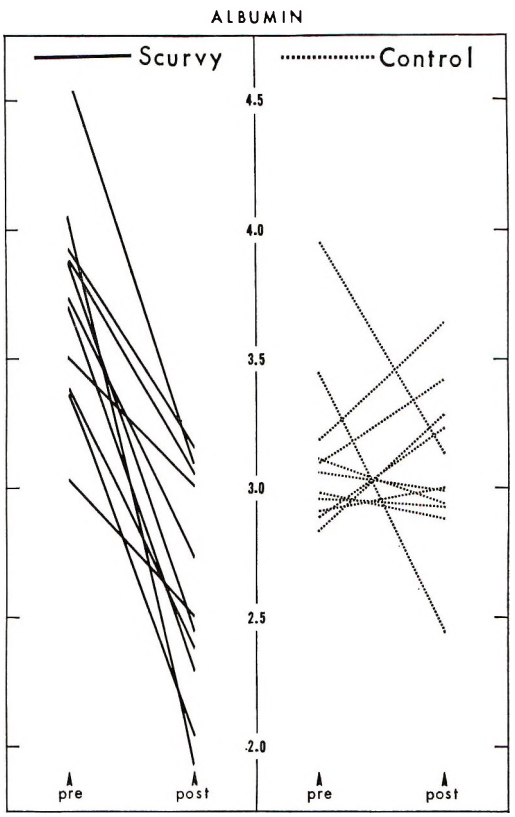


Fig. 1 Albumin serum protein values in grams per 100 milliliters pre- and postexperiment period. Two groups are represented. Scorbutic animals (solid lines) fed unsupplemented rabbit ration and normal animals (interrupted lines) fed rabbit ration supplemented with 4 mg of vitamin C.

No precipitation lines were observed in agar gel when the absorbed rabbit antiscorbutic guinea pig serum was reacted with individual scorbutic sera. This suggests that there were no specific antigenic determinants in the sera of the scorbutic animals.

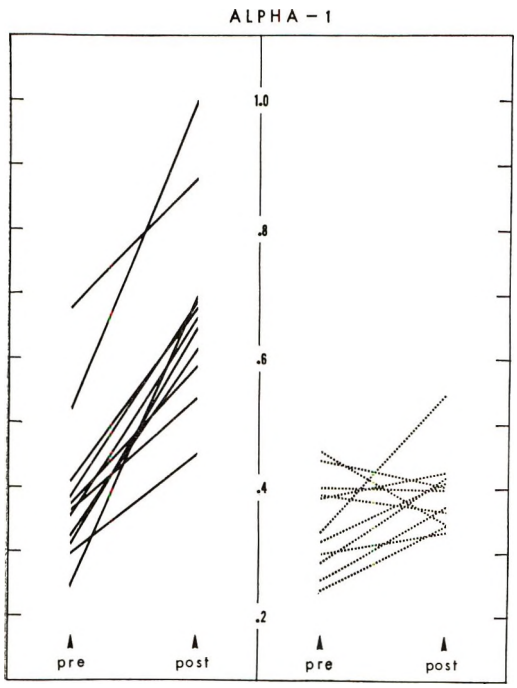


Fig. 2 Serum alpha-1 globulin values in grams per 100 milliliters pre- and postexperiment period. Two groups are represented. Scorbutic animals (solid lines) fed unsupplemented rabbit ration and normal animals (interrupted lines) fed rabbit ration supplemented with 4 mg of vitamin C.

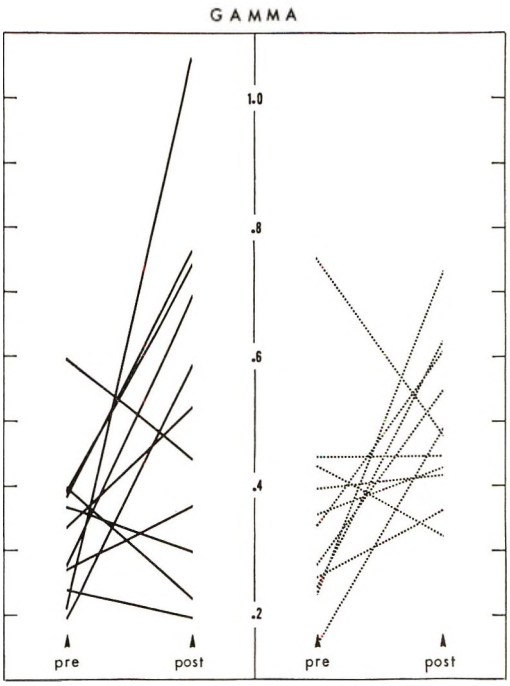


Fig. 3 Serum gamma globulin values in grams per 100 milliliters pre- and postexperiment period. Two groups are represented. Scorbutic animals (solid lines) fed unsupplemented rabbit ration and normal animals (interruped lines) fed rabbit ration supplemented with 4 mg of vitamin C.

In the present study, histological changes suggestive of amyloidosis did not develop. The studies of guinea pig amyloidosis produced by ascorbic acid depletion were described in animals having partial but incomplete vitamin C supplementation (2, 3). In these studies the animals failed to grow, became ill, but lingered for 3 to 4 months. Histological examination showed amyloid deposits.

Our own attempts to produce a partially supplemented chronic state were unsuccessful, either because the animals died, or because they failed to develop the disease. A group of guinea pigs was fed rabbit ration supplemented with 0.2 mg vitamin C/day (the amount used by Pirani et al. (2)). By 2 months, 8 out of 10 animals showed marked weight loss and died. Histological examination revealed hemorrhages but no evidence of amyloid. The remaining two animals were eventually killed, but no evidence of amyloidosis was found. In another group of animals supplemented with 0.4 mg/day, 17 out of 20 animals were still alive at the end of 5 months; they continued to grow and were completely normal.

Considerable variation in the susceptibility to scurvy was noted from animal to animal and it is not clear whether genetic or environmental factors are responsible for this variation.

LITERATURE CITED

1. Rhinehart, J. F., C. L. Connor and S. R. Mettier 1934 Further observations on pathologic similarities between experimental scurvy combined with infection, and rheumatic fever. J. Exp. Med., *59:* 97.
2. Pirani, C. L., C. G. Bly, K. Sutherland and F. Chereso 1949 Experimental amyloidosis in guinea pig. Science, *110:* 145.
3. Lautsch, E., and F. Gagne 1951 Experimental amyloidosis in vitamin C-deficient guinea pigs. Rev. Can. Biol., *10:* 119.
4. Lagier, R., and J. Monnier 1958 Electrophoretic changes of the serum in the scorbutic

guinea pig (partial deficiency and total deficiency); comparative study of certain inflammatory conditions (turpentine-induced abscess and tuberculosis). Rev. Franc. Etud. Clin. Biol., 3(4): 437.
5. Howard, A. N. 1958 Plasma proteins in vitamin C deficiency. Brit. J. Nutr., *12*: 346.
6. Banerjee, S., and K. Rohatgi 1958 Different fractions of serum proteins in normal and scorbutic guinea pigs. Proc. Soc. Exp. Biol. Med., *97*: 234.
7. Kabat, E. A., and M. M. Mayer 1961 Experimental Immunochemistry. C. C Thomas, Springfield, Illinois, p. 559.

Influence of Microorganisms on Intestinal Absorption: Oleic Acid ^{131}I and Triolein ^{131}I Absorption by Germfree and Conventionalized Rats [1,2]

BUD TENNANT, MARIO REINA-GUERRA, DORIS HARROLD AND MARVIN GOLDMAN

Department of Clinical Sciences and the Radiobiology Laboratory, School of Veterinary Medicine, University of California, Davis, California

ABSTRACT Several differences in lipid metabolism have been demonstrated between germfree and conventional rats. To evaluate the role which intestinal absorption might play in determining these differences, we compared the absorption of oleic acid ^{131}I and triolein ^{131}I in germfree and conventionalized rats. Gastric emptying of both compounds appeared to be delayed in germfree rats and correspondingly less radioactivity reached the cecum during the 6-hour period following intragastric administration. When corrections were made for differences in gastric emptying, germfree and conventionalized rats absorbed oleic acid and triolein at similar rates. Under the conditions of our studies, intestinal microorganisms did not appear to influence the rate of either lipolysis or fatty acid absorption directly, but significantly influenced the rate at which fat was transported along the gastrointestinal tract.

Investigations with germfree animals have demonstrated the important influences which intestinal microorganisms have on lipid metabolism (1–5). Total body fat was shown to be significantly lower in germfree rats than in conventionalized rats (1). Plasma cholesterol levels, however, frequently are higher in germfree rats than in conventional rats and the rate of cholesterol catabolism is significantly reduced in germfree rats (5–7). Bile acid turnover is three to five times more rapid in conventional rats and there are significant qualitative differences in the bile acids excreted by germfree and conventional rats (6, 8–10).

Evrard et al. (3), using a metabolic balance method, observed reduced utilization of dietary fat by conventional rats. Wiech et al. (4), however, found no differences in the intestinal absorption of linoleic acid between germfree and conventional rats but observed that the rate of oxidation of linoleic acid was reduced in germfree rats. We have conducted further studies to evaluate the importance of intestinal microorganisms on fat digestion and absorption. This report summarizes observations on the utilization of oleic acid ^{131}I and triolein ^{131}I by germfree and conventionalized rats.

MATERIALS AND EXPERIMENTAL PROCEDURES

Germfree, Fischer strain rats were obtained from a commercial source [3] at weaning and were transported to our laboratory in isolators designed for shipping. Upon arrival, they were transferred to a germfree Trexler-type flexible plastic isolator (11) where they were maintained until the age of 4 to 5 weeks. At that time, they were divided randomly according to sex and body weight into two equal groups. One of the groups was transferred into another plastic isolator and was conventionalized by a modification of a method previously described (1). Briefly, this procedure involved contamination of the drinking water with cecal contents ob-

Received for publication August 5, 1968.

1 This work was conducted as part of Western Regional Research Project, W-88 and was supported by RRF-2279.

2 A preliminary report of these results was presented at the annual meeting of the Association for Gnotobiotics, Incorporated, Buffalo, New York, June 9–10, 1968.

3 Charles River Breeding Laboratories, Wilmington, Massachusetts.

tained from a rat from an open-animal room. Two weeks after this initial step, open-animal-room rats were introduced into the cages of the conventionalized rats and were allowed to live in association with these animals for a 1-month period.

Both germfree and conventionalized rats were raised in isolators which were kept side by side and both isolators received essentially identical treatment. The rats were maintained in plastic cages, two to three to a cage, with pine wood shavings used as bedding. They were fed commercially prepared rations, diet L-356 [4] (12), and distilled water which were sterilized in an autoclave at 122° for 1 hour. A multivitamin preparation [5] was added to the drinking water at a rate of approximately 0.1 ml/100 ml of drinking water. The heat-sterilized food and water, the glass ampules containing the vitamin supplement and the glass ampules of radioactive oleic acid and triolein were entered into germfree and conventionalized isolators through peracetic acid-sterilized locks. The conventionalized isolator barrier was broken only when open-animal-room rats were entered and removed.

The microbiologic status of the isolators was evaluated at biweekly intervals using procedures similar to those described by Levenson and Tennant (1). All cultures from the germfree isolator were negative. No detailed attempts were made to characterize the microflora of the conventionalized rats. The fecal flora was complex, however, and, in addition to *Escherichia coli*, gram-positive rods and gram-positive and gram-negative cocci were demonstrated.

At 4.5 months of age, approximately equal numbers of male and female, germfree and conventionalized rats were transferred to separate, sterile experimental isolators. They were housed in individual stainless steel cages with false-bottom floors made of three-mesh galvanized wire. Food and water were withheld overnight and the following morning 1 μCi of oleic acid ^{131}I [6] in 0.2 ml of oleic acid carrier was administered using a tuberculin-type syringe attached to an intragastric tube.

Six hours after administration of the labeled oleic acid, the rats were killed with chloroform. The gastrointestinal tract was removed and divided into four sections: stomach, small intestine, cecum and colon. Each section, including its contents, was placed in a separate Pyrex glass counting tube and the amount of radioactivity determined in an automatic scintillation well counter [7] equipped with 8 cm by 8 cm sodium iodide (T1) detector and a single channel analyzer calibrated to count the 0.364 Mev photons of ^{131}I. Suitable aliquots of the test solution were also counted with the samples to provide data for correction of radioactive decay.

Triolein absorption was studied using procedures similar to those described above. The rats were 5 months of age when the experiment was conducted and 1 μCi of triolein ^{131}I [8] in 0.2 ml of peanut oil was used as the test material.

RESULTS

The percentages of triolein ^{131}I and oleic acid ^{131}I present in the gastrointestinal tract 6 hours following intragastric administration are given in table 1. The stomachs of the germfree rats contained 45.0 ± 9.4% [9] of the administered triolein ^{131}I whereas 23.4 ± 3.9% was present in the stomachs of the conventionalized rats ($P < 0.01$).[10] A similar trend was observed for oleic acid ^{131}I; germfree rats retained 36.0 ± 6.3% of the administered oleic acid ^{131}I in their stomachs, whereas the conventionalized rats retained 26.2 ± 4.2%. This latter difference was not, however, significant at the 0.05 level ($P = 0.1$).

[4] General Biochemicals Incorporated, Chagrin Falls, Ohio.

[5] Vi-Syneral, U. S. Vitamin and Pharmaceutical Corporation, New York, New York. Each milliliter of the preparation contained 5,000 USP units of vitamin A, 500 USP units of calciferol, 1 IU *dl*, α-tocopheryl acetate and the following concentrations of water-soluble vitamins in milligrams: ascorbic acid, 25; thiamine·HCl, 5; riboflavin, 0.5; niacinamide, 10; pyridoxine·HCl, 1.5; and panthenol, 2.5. The solution also contained 3% sorethytan laurate, 0.7% sodium hydroxide, 1% gentisic acid ethanolamide, and 0.5% chlorbutanol.

[6] The oleic acid ^{131}I and triolein ^{131}I used in these experiments were obtained from Abbott Laboratories, North Chicago, Illinois. Dilutions of the primary compounds were performed in our laboratory. Oleic acid ^{131}I was diluted with carrier oleic acid and the primary triolein ^{131}I was diluted with peanut oil so that the activity of each was 5 μCi/ml. These diluted materials were sealed in glass ampules which were then sterilized at 122° for 15 minutes prior to entry into the isolators through peracetic acid-sterilized locks.

[7] Baird-Atomic, Cambridge, Massachusetts.

[8] See footnote 6.

[9] SE.

[10] Student's *t* test was used to compare the significance of differences between means.

TABLE 1

Percentages of oleic acid ^{131}I and triolein ^{131}I in the gastrointestinal tract 6 hours following administration by gastric lavage

	Status	No. of rats	Stomach	Small intestine	Cecum	Colon
			%	%	%	%
Oleic acid ^{131}I	germfree	5	36.0 ± 6.3 [1]	8.7 ± 1.1	0.8 ± 0.4 [2]	0.1 ± 0.0 [3]
	conventionalized	4	26.2 ± 4.2	7.9 ± 1.9	3.5 ± 1.2	0.2 ± 0.1
Triolein ^{131}I	germfree	7	45.0 ± 9.4 [4]	9.9 ± 2.0	0.3 ± 0.0 [3,4]	0.1 ± 0.0 [3]
	conventionalized	7	23.4 ± 3.9	12.1 ± 2.8	4.4 ± 0.7	0.3 ± 0.1

[1] Mean ± SE.
[2] Difference between germfree and conventionalized rats significant ($P < 0.05$).
[3] Standard error was less than 0.05.
[4] Difference between germfree and conventionalized rats significant ($P < 0.01$).

No significant differences were observed between germfree and conventionalized rats in the amounts of either triolein ^{131}I or oleic acid ^{131}I found in the small intestines. In the germfree rats, 8.7 ± 1.1% of the oleic acid ^{131}I was recovered in the small intestine and 7.9 ± 1.9% was recovered in the small intestine of conventionalized rats. In the triolein ^{131}I trial, 9.9 ± 2.0% of the test dose was recovered in the small intestines of germfree rats and 12.1 ± 2.8% in the conventionalized rats.

Significant differences were observed between germfree and conventionalized rats in the amounts of both triolein ^{131}I and oleic acid ^{131}I found in the cecum. In germfree rats, 0.8 ± 0.4% of the administered oleic acid ^{131}I was recovered in the cecum compared with 3.5 ± 1.2% in conventionalized rats ($P < 0.05$). For triolein ^{131}I, 0.3 ± 0.03% of the dose was found in the cecum of germfree rats, whereas 4.4 ± 0.7% was found in the cecum of conventionalized rats ($P < 0.01$).

Small quantities of oleic acid ^{131}I and triolein ^{131}I were present in the colons of germfree and conventionalized rats and the differences between these groups were not significant. Only 0.1% of the oleic acid ^{131}I and triolein ^{131}I was present in the colons of germfree rats 6 hours after administration. In conventionalized rats, 0.2% of the oleic acid and 0.3% of the triolein were present in the colon.

To calculate the amounts of oleic acid ^{131}I and triolein ^{131}I actually absorbed by germfree and conventionalized rats it was necessary to correct for the differences which were observed in gastric emptying. To correct for this variable, we used the method of Mäkelä et al. (13) and assumed that only the amount of radioactivity which left the stomach (the total administered dose minus the amount recovered in the stomach) was actually available for absorption. The percentage absorbed was then calculated as that fraction of the radioactivity which was available for absorption but which was not recovered in the small intestine, cecum and colon. The percentages of oleic acid ^{131}I and triolein ^{131}I absorbed by germfree and conventionalized rats were essentially identical (table 2). Germfree rats absorbed 83.8 ± 2.7% of the oleic acid ^{131}I which cleared the stomach, whereas conventionalized rats absorbed 84.5 ± 3.0%. In the triolein ^{131}I study, germfree rats absorbed 77.7 ± 4.7% of the fat available for absorption and conventionalized rats absorbed 77.4 ± 3.9%.

DISCUSSION

Morphological studies suggest that the intestine of the germfree animal has significantly less mucosal surface area (14) and a smaller villus-to-crypt ratio (15) than conventional animals. On morphological criteria, one might predict that the intestinal absorptive capacity of germfree animals would be less than that of their conventional counterparts. Biochemical studies of intestinal absorption, however, have actually demonstrated the opposite of that which would be predicted on the basis of mucosal surface area; namely, that the intestinal absorptive capacity of germfree animals frequently is greater than that of conventional animals. Xylose

TABLE 2

Percent absorption of oleic acid ^{131}I and triolein ^{131}I 6 hours following gastric lavage

	Status	No. of rats	Body wt	Percent of dose absorbed [1]
			g	
Oleic acid ^{131}I	germfree	5	189.6 ± 15.9 [2]	83.8 ± 2.7
	conventionalized	4	194.8 ± 29.0	84.5 ± 3.0
Triolein ^{131}I	germfree	7	211.3 ± 16.9	77.7 ± 4.7
	conventionalized	7	208.9 ± 19.4	77.4 ± 3.9

[1] Corrections for differences in gastric emptying were made by calculating percent absorption as the difference between the administered dose and the percentage of the dose which was unabsorbed (13):

$$\text{Unabsorbed radioactivity} = \frac{a}{(A - B)} \times 100$$

where

a = radioactivity in small intestine, cecum and colon
A = radioactivity administered intragastrically
B = radioactivity retained in the stomach

[2] Mean ± SE.

(16), thiamine [11] and methionine [12] have all been shown to be absorbed more rapidly in germfree animals than in conventionals. The data of Evrard et al. (3) also suggest that dietary fat may be utilized more efficiently in germfree rats. The explanation for these observations is not completely clear. It has been suggested that the increased intestinal absorptive capacity of germfree animals is related to the decreased cellularity and thickness of the lamina propria (16). It is possible also that germfree and conventional animals differ in the functional capacity of their mucosal cells. It is known, for example, that the life span of the mucosal cell in germfree mice is significantly longer (15), and the age of the mucosal cell has been recognized as an important determinant of absorptive function (17).

The data of Wiech et al. (4) on the absorption of linoleic acid and our own observations on oleic acid absorption are quite comparable. In both studies, germfree rats appeared to absorb long-chain fatty acids at rates similar to those of rats with an intestinal microflora. These results, however, are different from those referred to above which indicated more rapid absorption of xylose (16), methionine [13] and thiamine [14] in germfree rats. We have no explanation for these differences but they suggest that the effects of microorganisms on intestinal absorption vary depending on the particular compound being studied. The fatty acid absorption data and the information on the absorption of xylose, methionine and thiamine when taken together suggest no correlation between the absorption rate of these compounds and the previously described data on mucosal surface area of germfree and conventional rats (14).

The absorption of triolein ^{131}I occurred at the same rates in germfree and conventionalized rats indicating that the triglyceride lipolysis rates were also similar. This is not to say that certain factors of primary importance in the hydrolysis of fat are not affected by intestinal microorganisms because it is known that bile acid metabolism is altered in significant ways by such microorganisms. Under the conditions of our studies, however, net intraluminal lipolysis of triglyceride did not appear to be influenced by intestinal microorganisms.

Gastric emptying of both oleic acid ^{131}I and triolein ^{131}I appeared to be significantly delayed in germfree rats and the amounts reaching the cecum during the test period were also significantly reduced. This indicates that gastrointestinal motility was significantly reduced in germfree rats, an observation similar to that of Abrams and Bishop (18). These data suggest that mi-

[11] Gordon, H. A., E. Bruckner-Kardoss and D. Kan 1960 Effects of normal microflora on structural and absorptive characteristics of the intestine. In: Abstracts of the 5th International Congress of Nutrition, September 1–7, Washington, D. C., p. 21.

[12] Herskovic, T., J. Katz, M. H. Floch, R. P. Spencer and H. M. Spiro 1967 Small intestinal absorption and morphology in germfree, monocontaminated, and conventionalized mice. Gastroenterology, 52: 1136 (abstract).

[13] See footnote 11.

[14] See footnote 12.

croorganisms have an important role in determining the normal propulsive activities of the gastrointestinal tract.

LITERATURE CITED

1. Levenson, S. M., and B. Tennant 1963 Some metabolic and nutritional studies with germfree animals. Federation Proc., 22: 109.
2. Tennant, B., O. J. Malm, R. E. Horowitz and S. M. Levenson 1968 Response of germfree, conventional, conventionalized and *E. coli* monocontaminated mice to starvation. J. Nutr., *94:* 151.
3. Evrard, E., P. P. Hoet, H. Eyssen, H. Charlier and E. Sacquet 1964 Faecal lipids in germfree and conventional rats. Brit. J. Exp. Pathol., *45:* 409.
4. Wiech, N. L., J. G. Hamilton and O. N. Miller 1967 Absorption and metabolism of dietary triglycerides in germfree and conventional rats. J. Nutr., *93:* 324.
5. Wostmann, B. S., N. L. Wiech and E. Kung 1966 Catabolism and elimination of cholesterol in germfree rats. J. Lipid Res., 7: 77.
6. Danielsson, H., and B. E. Gustafsson 1959 On serum-cholesterol levels and neutral fecal sterols in germfree rats. Bile acids and steroids 59. Arch. Biochem. Biophys., *83:* 482.
7. Wostmann, B. S., and N. L. Wiech 1961 Total serum and liver cholesterol in germfree and conventional male rats. Amer. J. Physiol., *201:* 1027.
8. Gustafsson, B. E., S. Bergström, S. Lindstedt and A. Norman 1957 Turnover and nature of fecal bile acids in germfree and infected rats fed cholic acid-24-^{14}C. Bile acids and steroids 41. Proc. Soc. Exp. Biol. Med., *94:* 467.
9. Gustafsson, B. E., A. Norman and J. Sjövall 1960 Influence of *E. coli* infection on turnover and metabolism of cholic acid in germfree rats. Arch. Biochem. Biophys., *91:* 93.
10. Gustafsson, B. E., and A. Norman 1962 Comparison of bile acids in intestinal contents of germfree and conventional rats. Proc. Soc. Exp. Biol. Med., *110:* 387.
11. Trexler, P. C. 1959 The use of plastics in the design of isolator systems. Ann. N. Y. Acad. Sci., *78:* 29.
12. Larner, J., and R. E. Gillespie 1957 Gastrointestinal digestion of starch. III. Intestinal carbohydrase activities in germfree and non-germfree animals. J. Biol. Chem., *225:* 279.
13. Mäkelä, T. E., J. Hakkila and M. Siurla 1962 The effect of triolein and oleic acid upon the absorption of labeled triolein-I^{131} and oleic acid-I^{131} in rats. Ann. Med. Exp. Biol. Fenn., *40:* 231.
14. Gordon, H. A., and E. Bruckner-Kardoss 1961 Effect of normal microbial flora on intestinal surface area. Amer. J. Physiol., *201:* 175.
15. Abrams, G. D., H. Bauer and H. Sprinz 1963 Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. Lab. Invest., *12:* 355.
16. Heneghan, J. B. 1963 Influence of microbial flora on xylose absorption in rats and mice. Amer. J. Physiol., *205:* 417.
17. Padykula, H. A., E. W. Strauss, A. J. Ladman and F. H. Gardner 1961 A morphologic and histochemical analysis of the human jejunal epithelium in nontropical sprue. Gastroenterology, *40:* 735.
18. Abrams, G. D., and J. E. Bishop 1967 Effect of the normal microbial flora on gastrointestinal motility. Proc. Soc. Exp. Biol. Med., *126:* 301.

Sequence of Limiting Amino Acids in Fish Protein Concentrate Produced by Isopropyl Alcohol Extraction of Red Hake (*Urophycis chuss*)

B. R. STILLINGS, O. A. HAMMERLE AND D. G. SNYDER
Bureau of Commercial Fisheries, Technological Laboratory, College Park, Maryland

ABSTRACT Experiments were conducted to determine the sequence of limitation of the essential amino acids in fish protein concentrate (FPC) produced by isopropyl alcohol extraction of whole red hake (*Urophycis chuss*). Diets were prepared containing 1.28% nitrogen from FPC and 0.32% nitrogen from various combinations of amino acids. The diets were fed to weanling rats for 4 weeks; and weight gain, feed intake and protein efficiency ratio were determined. The studies indicated that, as a nonessential nitrogen source, glutamic acid was utilized as efficiently as was a mixture of nonessential amino acids. From the results of these studies, the amino acids in FPC were grouped according to their limitation, from greatest to least, as follows: 1) methionine; 2) histidine, tryptophan and threonine; 3) valine, isoleucine and phenylalanine; and 4) leucine, lysine and arginine.

In recognition of the world need for dietary protein of high quality, attention is being focused on protein sources that can be used to supplement vegetable proteins of low quality. Animal proteins are generally higher in quality than vegetable proteins, because they contain ample quantities of the essential amino acids. Chemical analysis of the amino acids in protein supplements is not sufficient, however, to enable their true supplemental value to be assessed. Animal studies are also needed to determine the extent to which the amino acids are utilized. By conducting both chemical analyses and animal studies, the researcher can ascertain the amino acids that limit quality and the sequence in which they become limiting. With such information he can then combine protein sources to produce an amino acid pattern that more nearly corresponds to the requirements of the animal being fed.

One animal protein supplement with good potential for alleviating the world protein deficit is FPC (fish protein concentrate). A chemical process for producing FPC from red hake (*Urophycis chuss*) by the use of isopropyl alcohol to remove water and oil has recently been developed by the Bureau of Commercial Fisheries (1). Nutritional studies have shown that the quality of the protein of this product is comparable to that of milk protein (1, 2); however, no animal studies have been reported on the sequence of limiting amino acids in FPC.

Chemical analysis showed that the amino acids were well balanced in relation to the requirements of animals (2). Therefore, the range between the greatest and least limiting amino acid was expected to be narrow. When a protein is well balanced, different approaches must be used in a series of experiments to characterize the sequence of limiting amino acids. Accordingly, the sequence of the limiting amino acids in FPC prepared by isopropyl alcohol extraction of red hake was determined in a series of animal experiments.

PROCEDURES AND RESULTS

Experiment 1. Sequence of most-limiting amino acids

The aim of this experiment was to determine the limitation sequence of the amino acids in FPC that were in greatest deficit with respect to the amino acid requirements of the rat (3, 4). The amino acids studied were phenylalanine, isoleucine, tryptophan, threonine, histidine and methionine.

Materials and methods. The FPC was prepared from a single catch of red hake

Received for publication August 22, 1968.

by a three-stage cocurrent batch extraction with isopropyl alcohol (1). The batches weighed about 10 kg each. Four batches were blended to produce the sample used in this study.

The sample was analyzed for total nitrogen by the macro-Kjeldahl procedure (5). The amino acid composition was determined with an automatic amino acid analyzer after hydrolysis of the sample for 24 hours in 6 N HCl at 110°. Cystine in the sample was analyzed by a microbiological method; and tryptophan, by the method of Spies and Chambers (6).

Male rats of the Holtzman strain were obtained at 22 days of age and were fed a diet containing 15% casein for two days. The animals were then assigned to groups of 10, on a weight basis; and the groups were randomly assigned to diets. The animals were fed ad libitum for 28 days, and feed intake and weight gain data were recorded. The protein efficiency ratio (PER) was calculated by dividing the gain in weight by the crude protein consumed (nitrogen × 6.25).

A small percentage of animals was affected by enzootic viral pneumonia. To minimize the effect of this factor, data from only the 8 animals gaining the most weight in each group were analyzed.

Diets and design. The composition of the basal diet is shown in table 1. It was formulated to contain 1.28% nitrogen from FPC and 0.32% nitrogen from a mixture of nonessential amino acids. The mixture was similar to that used in diet BII by Ranhotra and Johnson (7), except that cystine and tyrosine were omitted from the mixture to avoid their interference in determining the limitations of methionine and phenylalanine.

TABLE 1

Composition of basal diet

	%
Nitrogen source [1] Dextrose [2]	70.0
Sucrose	10.0
Nonnutritive fiber [2]	5.0
Salt mixture USP XIV [2]	4.0
Vitamin fortification mix [2,3]	1.0
Corn oil [4]	10.0

[1] Fish protein concentrate furnished 1.28% nitrogen and crystalline nonessential amino acids supplied 0.32% nitrogen.

[2] Obtained from General Biochemicals, Inc., Chagrin Falls, O.

[3] Vitamin mix composition (mg/100 g diet): vitamin A, (dry, 500 IU/mg), 4.0; vitamin D_2, (dry, 500 IU/mg), 0.44; α-tocopherol acetate, (dry, 250 IU/g), 49; ascorbic acid, 100; inositol, 11; choline citrate, 370; menadione, 5.0; *p*-aminobenzoic acid, 11; niacin, 9.9; riboflavin, 2.2; pyridoxine·HCl, 2.2; thiamine·HCl, 2.2; Ca pantothenate, 6.6; biotin, 0.04; folic acid, 0.20; and vitamin B_{12}, 3.0 (0.1% in mannitol).

[4] Mazola, Corn Products Company, New York, N. Y.

The control diet was identical to the basal diet, except that the nonessential amino acids were replaced isonitrogenously with all six essential amino acids under study, that is, phenylalanine, isoleucine, tryptophan, threonine, histidine and methionine. The quantities of essential amino acids supplied by FPC (sample 1) and the amounts of the six crystalline amino acids added to the diet are shown in table 2. These amounts were selected to provide total dietary amino acid levels that were similar to those used in diet BII by Ranhotra and Johnson (7), which permitted excellent growth in rats.

Six experimental diets were used; in each diet one of the above-mentioned amino acids was omitted from the supplement.

Data were subjected to analysis of variance, and differences between means were determined by Tukey's procedure (8).

Results. Table 3 presents results obtained in this experiment. Removal of methionine from the supplement caused the greatest decrease in PER. Significantly lower PER values were also obtained when either histidine, threonine or tryptophan was removed from the supplement, but these three values differed little among themselves.

Significant depressions in weight gain resulted only when either histidine or methionine was lacking. When histidine was lacking, the lower weight gain reflected primarily a decreased intake of feed, as well as a lower efficiency of utilization. When methionine was lacking, the depressed weight gain was attributable only to the markedly lowered efficiency of utilization of feed protein.

On the basis of these results, the amino acids studied were grouped from most limiting to least limiting as follows: 1) methionine; 2) histidine, threonine and tryptophan; and 3) isoleucine and phenylalanine.

TABLE 2

Amino acid composition of fish protein concentrate (FPC) samples and supplements

Amino acid	Sample 1 [1]	Sample 2	Supplements [2]
	% of diet	*% of diet*	*% of diet*
Lysine (Lys)	0.661	0.638	0.250
Histidine (His)	0.157	0.155	0.100
Arginine (Arg)	0.529	0.512	0.200
Threonine (Thr)	0.347	0.359	0.150
Valine (Val)	0.411	0.414	0.140
Methionine (Met)	0.261	0.264	0.430
Cystine (Cys)	0.077	0.062	—
Leucine (Leu)	0.595	0.607	0.135
Isoleucine (Ile)	0.355	0.373	0.200
Tyrosine (Tyr)	0.256	0.239	—
Phenylalanine (Phe)	0.315	0.322	0.425
Tryptophan (Trp)	0.077	0.078	0.075

[1] Composition when FPC supplied 1.28% nitrogen to the diet. Both samples contained 14.3% nitrogen on a dry matter basis.

[2] Diets were supplemented with various combinations of the amino acids shown, plus nonessential nitrogen to equal 0.32% dietary nitrogen. All supplemental amino acids were the L form, NRC grade, obtained from General Biochemicals, Inc., Chagrin Falls, O. Lysine, histidine and arginine were added as the hydrochlorides.

TABLE 3

Effect of removing individual amino acids from supplements on weight gain, feed intake and protein efficiency ratio (exp. 1)

Dietary nitrogen source	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
1.28% FPC [1]-nitrogen, 0.32% nitrogen from 6 EAA and NEAA	3.50 ± 0.08 [2]	9.85 ± 0.21	3.51 ± 0.06
Same as above minus: [3]			
Phe	3.68 ± 0.08	10.19 ± 0.16	3.55 ± 0.10
Ile	3.46 ± 0.13	10.23 ± 0.48	3.35 ± 0.11
Trp	3.28 ± 0.17	10.44 ± 0.42	3.09 ± 0.07
Thr	3.62 ± 0.19	11.78 ± 0.44	3.01 ± 0.07
His	2.13 ± 0.05	7.04 ± 0.18	2.97 ± 0.05
Met	2.69 ± 0.15	10.32 ± 0.31	2.55 ± 0.09
Tukey's W ($P < 0.05$)	0.52	1.37	0.35

[1] FPC = fish protein concentrate. Essential amino acids (EAA) added were those shown in the table. Nonessential amino acid (NEAA) mixture had the same percentage composition as used in diet BII by Ranhotra and Johnson (7) except that cystine and tyrosine were not included.

[2] SE.

[3] Essential amino acids were replaced isonitrogenously with NEAA. For amino acid abbreviations, see table 2.

Experiment 2. Definition of first-limiting amino acid

Experiment 1 indicated that methionine was the first-limiting amino acid in FPC. The purpose of experiment 2 was to complement these results by a different experimental approach to define conclusively the first-limiting amino acid in FPC. In addition, to simplify formulation, we tested glutamic acid as a source of nonessential nitrogen in place of a mixture of nonessential amino acids.

Materials and methods. The materials and methods of analysis were the same as those described for experiment 1.

Diets and design. The control diet was identical to the basal used in experiment 1; that is, it contained 1.28% nitrogen from FPC and 0.32% nitrogen from a mixture of nonessential amino acids.

To determine the effectiveness of glutamic acid as a source of nonessential nitrogen, we included one diet that contained 0.32% nitrogen from glutamic acid instead

of from the nonessential amino acid mixture.

To determine the first-limiting amino acid, we supplemented each of six experimental diets with one of the following amino acids: phenylalanine, isoleucine, tryptophan, threonine, histidine or methionine.

Results. A significant increase in PER was obtained only when methionine was added to the diet (table 4). Also, significantly higher intakes of feed and gains in weight resulted from supplementation with methionine. Supplementation with histidine also increased feed intake and weight gain, but PER remained unchanged. These results and those from experiment 1 indicate that methionine is indeed the first-limiting amino acid.

Table 4 indicates results of the comparison between glutamic acid and the mixture of nonessential amino acids. Slightly higher weight gain and a similar PER were obtained with glutamic acid. We concluded, therefore, that glutamic acid could furnish nonessential nitrogen as effectively as could the nonessential amino acid mixture.

Experiment 3. Sequence among histidine, threonine and tryptophan

The data from experiments 1 and 2 showed that methionine was the first-limiting amino acid in FPC. Also, results from experiment 1 indicated that histidine, threonine and tryptophan were nearly equally second limiting. In experiment 3, we attempted to distinguish among these three amino acids with respect to their degree of limitation.

Materials and methods. The materials and methods used were the same as those used in experiment 1.

Diets and design. The basal diet was the same, except that nonessential nitrogen was supplied by glutamic acid instead of by the mixture of nonessential amino acids.

To determine the sequence of limitation of histidine, tryptophan and threonine, we used a 2^3 factorial design. The supplements used in the control and experimental diets contained methionine, since the previous two experiments showed that it was the first-limiting amino acid. Supplements for experimental diets also contained histidine, tryptophan and threonine in all possible qualitative combinations, in amounts as shown in table 2. Data were analyzed by the Yates' method of computing factorial effects, as described by Cochran and Cox (9), and by analysis of variance; and differences between means were determined by Tukey's procedure (8).

Results. A significant increase in PER was obtained only when all three amino

TABLE 4

Effect of adding individual amino acids to diets on weight gain, feed intake and protein efficiency ratio (exp. 2)

Dietary nitrogen source	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
1.28% FPC [1]-nitrogen, 0.32% nitrogen from NEAA	2.73 ± 0.20 [2]	9.77 ± 0.38	2.71 ± 0.10
Same as above plus: [3]			
Phe	2.81 ± 0.16	10.66 ± 0.38	2.59 ± 0.08
Ile	2.87 ± 0.15	10.72 ± 0.50	2.63 ± 0.08
Trp	2.81 ± 0.09	10.84 ± 0.26	2.55 ± 0.04
Thr	3.05 ± 0.10	10.77 ± 0.28	2.79 ± 0.04
His	3.16 ± 0.13	11.45 ± 0.45	2.69 ± 0.06
Met	3.59 ± 0.12	11.75 ± 0.29	3.03 ± 0.06
1.28% FPC-nitrogen, 0.32% nitrogen from glutamic acid	3.18 ± 0.11	11.64 ± 0.18	2.68 ± 0.08
Tukey's W ($P < 0.05$)	0.61	1.62	0.29

[1] FPC = fish protein concentrate. Mixture of nonessential amino acids (NEAA) with the same percentage composition as used in diet BII by Ranhotra and Johnson (7) except that cystine and tyrosine were not included.

[2] SE.

[3] Essential amino acids were added isonitrogenously at the expense of NEAA. For amino acid abbreviations, see table 2.

acids were added together (table 5). Factorial analysis of the main effects and interactions showed that both the influence on PER of tryptophan and threonine, and the interaction of histidine and tryptophan, were highly significant (table 6). Histidine and the interaction of histidine with either tryptophan or threonine had a significantly positive effect on feed intake and weight gain. A significantly negative effect on feed intake was obtained with tryptophan and threonine. Weight gain was also significantly reduced when only threonine was added to the diet.

Based on the responses in PER, a difference in limitation among histidine, threonine and tryptophan could not be distinguished. Factorial analysis showed, however, that each had a significant effect on PER; when they were added to the diet together, a significant increase in PER was obtained in comparison with the control. Therefore, these three amino acids were concluded to be nearly equally second limiting.

Experiment 4. Reevaluation of first- and second-limiting amino acids

Results from preceding experiments showed that methionine was the first-limiting amino acid in FPC and that histidine, threonine and tryptophan were nearly

TABLE 5

Effect of adding combinations of amino acids to diets on weight gain, feed intake and protein efficiency ratio (exp. 3)

Dietary nitrogen source	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
1.28% FPC [1]-nitrogen, 0.32% nitrogen from methionine and glutamic acid	4.78 ± 0.15 [2]	14.79 ± 0.40	3.20 ± 0.05
Same as above plus: [3]			
His	4.92 ± 0.14	15.49 ± 0.34	3.13 ± 0.03
Thr	4.40 ± 0.11	13.43 ± 0.41	3.33 ± 0.07
Trp	4.45 ± 0.13	13.55 ± 0.30	3.25 ± 0.04
His, Thr	4.83 ± 0.21	14.80 ± 0.51	3.23 ± 0.07
His, Trp	5.09 ± 0.16	14.98 ± 0.28	3.35 ± 0.07
Thr, Trp	3.72 ± 0.09	11.14 ± 0.36	3.29 ± 0.07
His, Thr, Trp	5.03 ± 0.08	14.04 ± 0.22	3.50 ± 0.05
Tukey's W ($P < 0.05$)	0.66	1.72	0.25

[1] FPC = fish protein concentrate.
[2] SE.
[3] Essential amino acids were added isonitrogenously at the expense of glutamic acid. For amino acid abbreviations, see table 2.

TABLE 6

Factorial main effects and interactions when diets were supplemented with amino acids [1] (exp. 3)

Amino acid [2]	Effect means		
	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
His	0.626 **	1.597 **	0.035
Trp	−0.162	−1.200 **	0.128 **
His, Trp	0.346 **	0.562 *	0.122 **
Thr	−0.313 **	−1.350 **	0.105 **
His, Thr	0.238 *	0.533 *	0.018
Trp, Thr	−0.078	−0.325	−0.008
His, Trp, Thr	0.093	0.201	0.032

[1] Calculated from data shown in table 5 by the Yates' method as given by Cochran and Cox (9).
[2] For amino acid abbreviations, see table 2.
* $P < 0.05$.
** $P < 0.01$.

equally second limiting. The same sample of FPC was used in all the previous studies. In recognition of the possibility of variation in the raw material, the purpose of experiment 4 was to determine if the first- and second-limiting amino acids in a second sample of FPC were the same as those in the sample used previously.

Materials and methods. The FPC was prepared from a different catch of red hake. The processing procedure and other conditions were the same as those described in experiment 1.

Diets and design. A 2^4 factorial design was used to determine the limitation sequence of methionine, histidine, threonine and tryptophan. No essential amino acids were added to the control diet, which was similar in amino acid composition to the basal diet used in experiment 3 (table 2). The experimental diets were supplemented with the four amino acids in all possible qualitative combinations, in the amounts shown in table 2.

Data were statistically analyzed by the same procedures as described in experiment 3.

Results. When the amino acids were added singly (table 7), methionine produced the largest, though statistically nonsignificant, response in PER. Compared with the value obtained when only methionine was included in the supplement, a significant additional response in PER was obtained only when all three of the other amino acids were supplemented. Factorial analysis (table 8) showed that methionine had the most significant effect on PER. Also, histidine, tryptophan and threonine, singly, and the interaction of tryptophan and threonine also had a highly significant positive effect on PER.

A highly significant increase in weight gain and in feed intake were obtained through the addition of histidine; however, a highly significant negative effect on feed intake resulted from the addition of only methionine and from the interaction of methionine, histidine and tryptophan.

The results from this study generally agreed with those from the previous experiments: Methionine was the first-limiting amino acid, and histidine, tryptophan

TABLE 7

Effect of adding combinations of amino acids to diets on weight gain, feed intake and protein efficiency ratio (exp. 4)

Dietary nitrogen source	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
1.28% FPC [1]-nitrogen, 0.32% nitrogen from glutamic acid	4.18 ± 0.12 [2]	14.20 ± 0.45	2.84 ± 0.02
Same as above plus: [3]			
Met	3.90 ± 0.08	12.75 ± 0.30	3.00 ± 0.04
His	4.15 ± 0.14	13.95 ± 0.42	2.93 ± 0.03
Trp	3.62 ± 0.23	12.79 ± 0.63	2.70 ± 0.05
Thr	3.97 ± 0.10	13.65 ± 0.35	2.83 ± 0.04
Met, His	4.21 ± 0.17	13.66 ± 0.40	3.05 ± 0.06
Met, Trp	3.72 ± 0.14	12.21 ± 0.35	2.97 ± 0.06
Met, Thr	3.75 ± 0.23	12.04 ± 0.60	3.02 ± 0.06
His, Trp	4.29 ± 0.17	14.21 ± 0.43	2.98 ± 0.04
His, Thr	3.71 ± 0.08	13.30 ± 0.15	2.76 ± 0.06
Trp, Thr	3.87 ± 0.16	12.71 ± 0.37	2.97 ± 0.05
Met, His, Trp	4.39 ± 0.19	13.40 ± 0.32	3.25 ± 0.09
Met, His, Thr	4.65 ± 0.19	14.01 ± 0.40	3.30 ± 0.07
Met, Trp, Thr	3.94 ± 0.12	12.03 ± 0.27	3.19 ± 0.04
His, Trp, Thr	4.30 ± 0.12	14.36 ± 0.35	3.05 ± 0.04
Met, His, Trp, Thr	4.52 ± 0.12	12.89 ± 0.35	3.48 ± 0.09
Tukey's W ($P < 0.05$)	0.77	1.97	0.31

[1] FPC = fish protein concentrate.
[2] SE.
[3] Essential amino acids were added isonitrogenously at the expense of glutamic acid. For amino acid abbreviations, see table 2.

TABLE 8

Factorial main effects and interactions when diets were supplemented with amino acids [1] (exp. 4)

Amino acid [2]	Effect means		
	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
Met	0.109	−0.774 **	0.274 **
His	0.421 **	0.922 **	0.158 **
Met, His	0.196 *	0.308	0.064 *
Trp	0.026	−0.371	0.105 **
Met, Trp	−0.016	−0.112	0.019
His, Trp	0.189 *	0.356	0.070 *
Met, His, Trp	−0.180 *	−0.558 **	−0.014
Thr	0.042	−0.275	0.109 **
Met, Thr	0.115	0.009	0.069 *
His, Thr	0.014	0.106	−0.019
Met, His, Thr	0.110	0.074	0.072 *
Trp, Thr	0.131	0.116	0.087 **
Met, Trp, Thr	−0.117	−0.202	−0.044
His, Trp, Thr	−0.075	−0.132	−0.033
Met, His, Trp, Thr	−0.095	−0.217	−0.023

[1] Calculated from data shown in table 7 by the Yates' method as given by Cochran and Cox (9).
[2] For amino acid abbreviations, see table 2.
* $P < 0.05$.
** $P < 0.01$.

and threonine were nearly equally second limiting.

Experiment 5. Sequence of least-limiting amino acids

In the previous experiments, the amino acids in FPC that were most limiting were studied. The aim of experiment 5 was to determine the sequence of limitation of those amino acids in FPC that were least limiting. These amino acids were arginine, lysine, leucine, phenylalanine and valine.

Materials and methods. The materials and methods were the same as those used in experiment 1.

Diets and design. The control diet was similar to the one used in experiment 3, except that it was supplemented with all 10 essential amino acids. Five experimental diets were used; one amino acid was omitted from each of the dietary supplements.

Results. Removal of arginine, lysine or leucine caused no significant decrease in PER (table 9), although the absence of leucine significantly reduced feed intake and weight gain. When either phenylalanine or valine was moved from the supplement, the PER and weight gain were significantly depressed; all values obtained when the supplement lacked valine were significantly less than were those obtained when phenylalanine was lacking.

On the basis of these results, the amino acids investigated in this experiment were grouped, from more limiting to less limiting, as follows: 1) valine and phenylalanine and 2) leucine, lysine and arginine.

DISCUSSION

The aim of these studies was to determine the sequence in which the essential amino acids in FPC are limiting. Since FPC produced from whole fish by isopropyl alcohol extraction has been shown to contain high quality protein (2), it was not expected that these studies would show a major deficiency of any of the amino acids. In fact, on a comparable protein basis, the amount of each essential amino acid in FPC is at least 80% of the requirement level for growth of the rat as given by NRC (3) and by Rama Rao et al. (4). In determining the limiting amino acids by comparing chemical composition with published requirement levels, however, certain variables are not taken into account. These include the degree of digestion and utilization of each amino acid, the accuracy of the requirement values, and the balance of amino acids in the protein. The studies re-

TABLE 9

Effect of removing individual amino acids from diets on weight gain, feed intake and protein efficiency ratio (exp. 5)

Dietary nitrogen source	Avg daily wt gain	Avg daily feed intake	Protein efficiency ratio
	g	*g*	
1.28% FPC [1]-nitrogen, 0.32% nitrogen from 10 essential amino acids and glutamic acid	6.12 ± 0.23 [2]	15.00 ± 0.45	4.00 ± 0.06
Same as above minus: [3]			
Arg	6.05 ± 0.11	15.04 ± 0.35	3.96 ± 0.04
Lys	5.95 ± 0.16	15.19 ± 0.31	3.84 ± 0.03
Leu	4.85 ± 0.15	12.52 ± 0.30	3.81 ± 0.05
Phe	5.15 ± 0.18	14.20 ± 0.43	3.57 ± 0.04
Val	4.09 ± 0.18	12.01 ± 0.45	3.36 ± 0.05
Tukey's W ($P < 0.05$)	0.73	1.64	0.20

[1] FPC = fish protein concentrate.
[2] SE.
[3] Essential amino acids were replaced isonitrogenously with glutamic acid. For amino acid abbreviations, see table 2.

ported here were conducted, therefore, to obtain more precise information on the limiting amino acids in FPC.

In evaluating the results of these studies, we gave primary emphasis to the efficiency of utilization of dietary nitrogen as measured by PER. Changes in weight gain may reflect differences in both feed intake and efficiency of utilization; however, at a specified dietary nitrogen level, PER should be affected primarily by the nutrient content of the diet. We recognize that dietary imbalances of specific amino acids can affect feed intake and that the PER method has its limitations as a precise measurement of efficiency of utilization; however, excellent agreement between this method and the net protein utilization method has been reported (10, 11), and PER was concluded to be an adequate method for these studies.

In designing these experiments to determine the limiting amino acid sequence in FPC, we divided the 10 essential amino acids in two groups. The first group of six contained those amino acids considered likely to be most limiting; the second group of four, least limiting. Addition of the first group of amino acids to the diet increased the PER from 2.7 to 3.5. When both groups were added to the diet, the PER was further increased to 4.0. Therefore, these studies were designed to determine which amino acids caused the increases in PER and to rank them in their order of limitation.

To find methionine the first-limiting amino acid in FPC was not surprising. Yanez et al. (12) reported that the sulfur-containing amino acids were first limiting in the FPC used in their study. In addition, the sulfur amino acids have been reported to be first limiting in fish meal (13, 14). Comparison of the amino acid composition of FPC with the requirements for amino acids (3, 4) showed that the sulfur amino acids were among the most likely to be first limiting.

Results from experiments 1, 3 and 4 showed that histidine, tryptophan and threonine were nearly equally second limiting. A significant response in PER was obtained only when all three were added along with methionine. In all experiments, addition of histidine had a stimulatory effect on feed intake and, consequently, on weight gain, but histidine by itself did not significantly influence PER.

Apparently valine, isoleucine and phenylalanine were nearly equally third limiting. In experiment 1, removal of phenylalanine from the supplement had no effect, and removal of isoleucine had only a slight effect, on PER. Thus, these two amino acids did not contribute significantly to the increased PER obtained when FPC was supplemented with the six amino acids. Removal of phenylalanine and valine from the supplement resulted in the greatest depression in PER (table 9). The large de-

pression in weight gain observed when valine was removed may have been a result of an antagonism between the branch-chained amino acids. These results indicated that valine, isoleucine and phenylalanine were limiting in the same order of magnitude, but no attempt was made to determine their order of limitation precisely.

Leucine, lysine and arginine appeared to be the least limiting amino acids in FPC. Removal of any one of these from the supplement did not significantly reduce PER (table 9).

From the results obtained with two different samples of FPC prepared by the chemical extraction of hake with isopropyl alcohol (1), the amino acids can be grouped according to their limitation, from greatest to least, as follows: 1) methionine; 2) histidine, tryptophan and threonine; 3) valine, isoleucine and phenylalanine; and 4) leucine, lysine and arginine.

ACKNOWLEDGMENT

McDonald Briscoe gave valuable technical assistance during these studies.

LITERATURE CITED

1. Bureau of Commercial Fisheries 1966 Marine protein concentrate, Fishery Leaflet 584. U. S. Department of the Interior, Washington, D. C.
2. Stillings, B. R. 1967 Nutritional evaluation of fish protein concentrate. In: Activities Report, vol. 19. Research and Development Associates, Inc., Natick, Mass., p. 109.
3. National Research Council 1962 Nutrient Requirements of Laboratory Animals. National Academy of Sciences — National Research Council, Washington, D. C.
4. Rama Rao, P. B., H. W. Norton and B. C. Johnson 1964 The amino acid composition and nutritive value of proteins. V. Amino acid requirements as a pattern for protein evaluation. J. Nutr., 82: 88.
5. Association of Official Analytical Chemists 1965 Official Methods of Analysis, ed. 10. Washington, D. C.
6. Spies, J. R., and D. C. Chambers 1949 Chemical determination of tryptophan in proteins. Anal. Chem., *21:* 1249.
7. Ranhotra, G. S., and B. C. Johnson 1965 Effect of feeding different amino acid diets on growth rate and nitrogen retention of weanling rats. Proc. Soc. Exp. Biol. Med., *118:* 1197.
8. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York.
9. Cochran, W. G., and G. M. Cox 1957 Experimental Designs, ed. 2. John Wiley and Sons, Inc., New York.
10. Henry, K. M. 1965 A comparison of biological methods with rats for determining the nutritive value of proteins. Brit. J. Nutr., *19:* 125.
11. Bressani, R., J. E. Braham, L. G. Ellias and S. DeZaghi 1965 Relationship between net protein utilization (NPU) and nitrogen efficiency ratio (NER). Nutr. Dieta, 7: 161.
12. Yanez, E., I. Barja, F. Moncheberg, A. Maccioni and G. Donoso 1967 The fish protein concentrate story. 6. Quintero fish protein concentrate: Protein quality and use in foods. Food Technol., *21:* 60.
13. Miller, D. S. 1956 The nutritive value of fish proteins. J. Sci. Food Agr., 7: 337.
14. Miller, E. L., and K. J. Carpenter 1964 Availability of sulphur amino acids in protein foods. I. Total sulphur amino acid content in relation to sulphur and nitrogen balance studies with the rat. J. Sci. Food Agr., *15:* 810.

Further Studies on Growth and Feed Utilization in Progeny of Underfed Mother Rats [1,2]

BOON-NAM BLACKWELL, R. QUENTIN BLACKWELL, THOMAS T. S. YU, YIH-SHYONG WENG AND BACON F. CHOW

Department of Biochemistry, U. S. Naval Medical Research Unit no. 2, Taipei, Taiwan, Republic of China, and Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland

ABSTRACT Previous studies have demonstrated growth-stunting and metabolic derangements, including reduced feed efficiency, in progeny of McCollum strain rat dams restricted to 50% dietary intake during gestation and lactation. These effects now have been duplicated in rats of the Sprague-Dawley strain. In addition, fostering techniques have allowed separate evaluation of the effects of maternal dietary restriction during gestation alone, and their comparison with the effect produced during the combined periods of gestation and lactation. Our results show, as suggested in earlier works, that restriction during gestation alone has a similar though perhaps lesser effect than restriction during gestation and lactation.

The important influence of maternal diet of the rat upon the ultimate growth and metabolic patterns of the offspring has been demonstrated by Chow and co-workers (1–4). It was found that dietary restriction of female rats during both gestation and lactation as well as during gestation alone was followed by permanent growth-stunting and metabolic derangement of the progeny fed ad libitum. Because of the implications of these findings to human nutrition, particularly with respect to possible means of improving the nutritional efficiency of populations in underdeveloped countries, further studies of the same general phenomenon were undertaken with several objectives in mind. First, it was considered essential to reproduce the original experiments (1) in another laboratory with a different strain of rats. Second, it was of interest to observe, by means of fostering techniques, effects on the offspring induced by maternal dietary deprivation during the gestation period alone, and to compare the effects with those arising from maternal dietary restriction during the combined periods of gestation and lactation. Third, effects of varying the litter size suckled by natural or foster mothers were studied.

EXPERIMENTAL METHODS

Animals. The rats used in this study were a Sprague-Dawley strain from the NAMRU-2 Biochemistry Department colony established in 1965 from animals obtained from the colony of the National Institutes of Health, Bethesda, and maintained with a commercial laboratory ration.[3]

Selection of experimental mothers. To obtain the most stable and effective mothers for the study, which in some cases was to include switching pups to foster mothers, a trial breeding was carried out for selection purposes. At the same time the initial pregnancy with its tendency for greater variability of the offspring was completed before the actual maternal diet study was begun.

Fifty 3-month-old virgin females were selected for uniform size; they were bred simultaneously, observed during pregnancy and delivery, and their litters inspected for number and birth weights. All litters were culled at birth to eight pups and the litters observed during approximately 1 week of

Received for publication December 9, 1967.

[1] This work was accomplished under the Bureau of Medicine and Surgery Work Unit MR005. 09-0076 and was supported in part by funding from the Advanced Research Projects Agency (Project AGILE) monitored by the Nutrition Section, Office of International Research, NIH/HEW, under ARPA Order 580, Program Plan no. 298, and in part by the Bureau of Medicine and Surgery, Department of the Navy, Washington, D. C.

[2] The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the U. S. Navy Department or the U. S. Naval Service at large.

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

lactation. The pups were all killed and the satisfactory mothers, totaling 44, were used for the maternal nutrition study. The remaining six females were rejected for bad temperament, tendency toward cannibalism and unsatisfactory litters.

Experimental feeding procedure. After the selection period and before the next breeding cycle the 44 selected females were allowed a 3-week rest period during which time they continued their consumption of commercial laboratory ration ad libitum. Next they were divided into mating groups of three each and one male of equal age was placed with each group. When morning vaginal lavage inspection for sperm was positive for individual females they were placed in separate cages and randomly assigned, from sets of five animals impregnated during the same night, one to each of five groups. Forty females, impregnated within 4 days, made up the final experimental groups with eight females in each of five groups. Group A consisted of females on unrestricted intake of commercial laboratory ration with daily dietary intake measured. In groups B1 and B2, the 16 females were restricted to 50% of the ad libitum intake of individual rats in group A; this dietary restriction was maintained throughout the pregnancy and lactation periods. Groups C1 and C2, like group A, were on unrestricted intake but no daily measurement of their intake was made. At birth all litters in groups A and B1 were reduced to eight pups each and their growth observed. At birth, four pups were taken from each mother in group B2 and given to a mother in group C1 which had delivered at almost the same time. Offspring from mothers in group C1 were not used. By this arrangement, each mother in group C1, fed an unrestricted diet, suckled four offspring from a group B2 mother. Each group B2 mother was left with four of her own offspring. The group C2 mothers, like those in group A, were each allowed to keep eight of their own offspring.

After weaning, at 21 days, all offspring were caged individually and allowed to feed ad libitum on the regular commercial laboratory ration.

Assessment of progeny. Four groups of progeny were obtained: Groups NN8, RR8, RR4 and RN4. Group NN8 included all animals from the 16 mothers fed ad libitum in groups A and C2; in all litters eight pups were maintained. Group RR8 contained offspring from the eight females in group B1 which were restricted to 50% of the dietary intake of group A mothers during pregnancy and lactation and which nursed eight pups per litter. The group RR4 progeny were from the eight group B2 mothers, with 50% maternal dietary restriction identical to that of group B1; however, in this group, each dam had nursed only four pups. The group RN4 progeny were from restricted B2 mothers but fostered, in litters of four, by group C1 mothers.

All progeny were observed for both feed intakes and body weights; feed intakes were determined continuously and body weights were measured at weekly intervals during the rapid-growth period and less frequently as the growth plateau was approached. In this report, observations during the first year are recorded.

RESULTS AND DISCUSSIONS

Survival. Among the 192 progeny, 4 died during the 21-day lactation period; all 4 were from RR8 litters. The remaining 188 pups including the 32 fostered pups in the RN4 group, survived the lactation period. For the remainder of the first year of life the respective number of deaths among progeny groups NN8, RR8, RR4 and RN4 were 4, 4, 3 and 1. One-year-survival rates for the four groups of progeny, in the same order, are 93.8, 87.5, 90.6 and 96.9%.

Growth. Mean and standard deviation values for body weights of progeny in the four groups at intervals during the 51-week period are summarized for male rats in table 1 and for the females in table 2. The mean body weight values in both tables were calculated from weights of individual animals surviving the entire period of 51 weeks. All progeny were on ad libitum diets of commercial laboratory ration. Comparison of growth of normal progeny from unrestricted mothers with those from mothers restricted during both gestation and lactation is provided by animals in groups NN8 and RR8. Table 1 shows the mean growth rates of those two groups of male progeny.

TABLE 1

Summary of body weights of male progeny

Age	Maternal diet groups			
	NN8 (8; 21) [1]	RR8 (8; 30)	RR4 (7; 12)	RN4 (8; 16)
weeks				
3	40.6± 3.3 [2]	22.5± 3.3	35.7± 4.3	42.9± 5.7
4	73.4± 6.7	42.0± 7.8	68.1±10.1	72.4± 9.9
5	116.4±13.1	74.8±11.1	110.2±18.2	114.2±14.6
6	165.8±14.5	107.8±16.6	141.6±24.9	144.8±18.7
8	250.3±18.8	179.5±23.8	219.9±35.6	226.8±22.8
10	311.0±23.3	234.5±31.1	270.7±35.0	287.2±25.4
12	345.3±25.0	272.3±35.2	312.3±36.4	322.7±23.4
14	372.9±28.3	298.3±36.5	343.8±38.6	346.9±26.0
16	383.5±31.1	309.8±36.1	358.9±39.3	360.6±29.1
19	401.1±35.0	325.1±36.3	373.2±39.4	373.7±31.2
22	409.7±38.7	333.2±36.4	382.2±37.6	380.6±33.2
25	419.9±41.0	344.5±39.4	392.8±38.0	395.4±37.9
28	427.9±35.6	355.1±38.2	393.9±36.6	396.1±36.2
32	436.4±34.6	366.6±37.0	412.3±33.3	413.5±34.0
36	425.8±40.2	357.0±35.8	401.9±34.0	400.2±34.7
41	414.7±47.2	357.0±34.3	394.8±35.4	398.1±32.8
46	440.3±48.5	364.3±40.0	420.8±29.5	412.6±31.1
51	460.1±48.9	374.0±46.0	436.4±34.5	419.6±30.8

[1] Numbers in parentheses indicate (number of litters; total number of males).
[2] Mean ± SD.

TABLE 2

Summary of body weights of female progeny

Age	Maternal diet groups			
	NN8 (8; 39) [1]	RR8 (8; 26)	RR4 (7; 17)	RN4 (8; 15)
weeks				
3	39.3± 4.4 [2]	20.5± 2.2	32.4± 3.7	40.1± 6.8
4	67.6± 7.3	39.8± 7.4	60.8± 8.8	66.0±10.6
5	107.5±10.7	69.5±11.0	94.5±18.7	98.7±16.4
6	142.3±11.7	96.2±15.7	120.1±19.8	125.7±19.4
8	184.6±15.0	137.4±16.0	158.1±22.6	165.6±19.7
10	210.0±25.1	164.1±17.9	183.0±30.2	191.0±20.3
12	230.5±18.2	184.7±18.6	203.4±25.2	204.9±20.8
14	242.9±19.8	196.5±18.1	214.0±23.6	218.4±20.5
16	250.3±20.4	205.1±17.9	221.2±21.2	224.6±18.9
19	259.2±21.4	219.9±26.6	231.4±25.5	231.9±17.9
22	262.6±21.9	221.2±21.1	235.7±24.8	236.2±16.2
25	265.9±23.1	225.5±19.1	231.6±35.3	232.1±18.6
28	271.0±23.6	230.2±18.4	240.6±24.7	241.4±21.6
32	285.0±22.1	241.2±17.1	251.2±29.5	253.7±22.1
36	279.0±23.6	236.3±18.8	248.6±25.9	248.4±20.3
41	291.5±27.0	245.1±17.7	263.2±23.0	259.7±20.4
46	298.1±33.6	246.1±19.0	263.2±27.0	262.9±24.8
51	309.9±35.5	258.1±19.0	268.9±22.9	269.0±20.7

[1] Numbers in parentheses indicate (number of litters; total number of females).
[2] Mean ± SD.

The 21 males in group NN8, from unrestricted mothers, grew at a substantially greater rate than the 30 males in the RR8 group. The stunting effect was seen in both male and female progeny. This is further illustrated by considering the mean body weight ratios for progeny groups at various age intervals. For example, the RR8/NN8

ratio for male progeny was 0.553 at 3 weeks of age and gradually increased to 0.813 at 51 weeks. The corresponding RR8/NN8 ratio for females began at 0.522 at 3 weeks and increased to 0.833 at 51 weeks.

These results, obtained in rats of the Sprague-Dawley strain, agree with those found previously by Chow and Lee (1) in rats of the McCollum strain. It appears to be clearly demonstrated by both studies that maternal dietary restriction during pregnancy and lactation produced permanent growth-stunting in the progeny.

Growth data for the progeny in groups RR4 and RN4 provide information relevant to the important question of the relative effect on progeny of maternal dietary restriction imposed during gestation versus that imposed during lactation. Data in table 1 demonstrate that males in the two groups grew at essentially the same rate; table 2 indicates that the same type of results was obtained for females in the two groups. Again, simple comparison of the growth rates is supplied by the ratios of mean body weights of the two groups. Except for the weaning weight ratios at 3 weeks, the RR4/RN4 ratios for both sexes were found to be within approximately 5% of unity for the entire 51-week period. In the progeny of both sexes no lasting benefit was derived from their transfer at birth from their own diet-restricted mothers to be suckled by normal foster mothers. There appeared to be an early slight transitory advantage for the RN4 groups as indicated by the mean body weights at weaning; however, this effect disappeared rapidly. Both males and females in the two groups had growth rates inferior to those of the corresponding normal, NN8, groups.

These data indicate that the stunting effect is induced in the progeny during gestation because the animals left in groups of four with their own diet-restricted mothers during the lactation period grew equally as well during the subsequent year as litter-mates fostered from birth by normally fed dams. If dietary restriction during pregnancy has no damaging effect on the offspring, one can expect that the RN4 animals should grow at least to normal size, or even bigger. The fact, however, is that RN4 animals and RR4 animals are the same size, and both are smaller than NN8 animals. The data suggest further that the state of restriction during lactation is relatively unimportant when the litter size is reduced to four. Both the dams fed restricted diet and those with normal diet appeared to have nearly equal ability to provide nutriture to their respective litters of four sucklings under the existing conditions of reduced demand resulting from small litter size. Although the progeny in both groups RR4 and RN4 were suckled in litters of four they did not achieve the above normal growth characteristically expected of such an arrangement (5–7). They grew better than the animals in group RR8, however, which could be taken as their standard for comparison.

Instead of growing faster than those in group NN8, the progeny in groups RR4 and RN4 grew at a rate distinctly below normal, and intermediate between the NN8 and RR8 rats. It appears particularly significant that the temporary weight advantage possessed by male and female progeny in the RN4 groups at weaning did not persist. This particular phenomenon has been repeatedly observed in other experiments in our laboratory. Clearly the progeny of diet-restricted mothers received damage during gestation which was not reversed by abundant nutriture during the lactation period.

These results, which indicate the definite influence of dietary restriction during gestation, support the earlier findings of Chow and Lee (1). In those studies, the maternal dietary restriction was imposed during mating and gestation only and the dams were returned to ad libitum feeding at parturition. Under these conditions the progeny showed the typical growth-stunting seen in the usual procedure of restriction during both gestation and lactation; however, it was uncertain under such stress conditions what period of ad libitum feeding would be required to replace the previously depleted reserves in the dams and how rapidly it would return them to normal. The present fostering technique for the progeny in group RN4 avoided that uncertainty.

The growth rates of both groups, RR4 and RN4, male and female, were greater than those of the corresponding RR8 groups. The mean body weight ratios,

RR4/RR8, for the male progeny groups began at approximately 1.6 in weeks 3 and 4 and diminished steadily to approximately 1.15 at 10 weeks after which they remained relatively constant. The ratios for RN4/RR8 in the male progeny behaved similarly. The corresponding ratios for the female progeny, RR4/RR8 and RN4/RR8, were approximately 1.53 and 1.66 at weaning and both decreased to values of approximately 1.10 at 12 weeks and 1.04 at 51 weeks. These ratios illustrate the temporary effects of improved nutriture during lactation.

Feed consumption. As reported previously (3) progeny from underfed mothers of the McCollum strain exhibited smaller gains in body weight per unit of feed consumed. This reduced feed efficiency was more marked in males and persisted in the adult animals. Feed efficiencies of male progeny from mothers with unrestricted diets were consistently better than those from restricted mothers. In the present study feed intakes were measured continuously for the entire 51-week period in all animals to determine possible differences in feed consumption and utilization.

The present results, which will be discussed and interpreted elsewhere along with similar data from other studies, show that the feed efficiencies for the NN8 progeny, both male and female, were higher than those of the corresponding RR8 groups; however, the differences were not as marked as those reported in rats of the McCollum strain (3). Animals from diet-restricted mothers had subnormal feed efficiencies which were most pronounced in the first 12 weeks of life during the most active period of growth. Temporary early improvement in feed efficiencies was provided to those progeny of underfed dams nursed in litters of four; that period of advantage allowed them to attain body weights intermediate between normal and the usual stunted size.

Tables 3 and 4 provide summaries of the comparisons of growth and feed utilization among progeny groups and illustrate the superiority of the normal progeny. As shown in table 4 a longer time and more total feed were required for the progeny of restricted dams to reach body weights achieved by the normal group in 12 weeks. Both male and female progeny of group NN8 had attained approximately 75% of their first year's growth at 12 weeks of age with mean weights of 345 g for males and 230 g for the females. By contrast males in the RR8 group required 25 weeks and 2.17 times as much feed to attain the 345 g size and the female RR8 group required 28 weeks and consumed 2.59 times the normal feed to reach 230 g.

CONCLUSIONS

Permanent effects of growth stunting and impaired feed utilization were pro-

TABLE 3

Comparison of growth rates and feed consumption

Group	No. of litters	No. of animals	Proportion of total mean growth for 51 weeks achieved during first 12 weeks	Total mean feed consumption / Mean body weight gain		
				First 12 weeks	13–51 weeks	Entire 51 weeks
			Males			
NN8	8	21	0.750	3.41	50.5	15.2
RR8	8	30	0.728	3.42	50.4	16.3
RR4	7	12	0.716	3.48	46.3	15.7
RN4	8	16	0.769	3.41	56.5	15.7
			Females			
NN8	8	39	0.744	4.18	62.2	19.1
RR8	8	26	0.716	4.24	61.2	18.8
RR4	7	17	0.757	4.16	70.6	20.3
RN4	8	15	0.762	4.23	70.7	20.1

TABLE 4

Comparison of relative periods of time and feed consumption required to attain specific body weights

Group	No. of litters	No. of animals	Age	Mean body wt	Total mean feed consumed	Total mean feed consumed / Mean body wt	Relative feed consumption to attain weight equal to that reached by the NN8 group at 12 weeks
			weeks	*g*	*g*		
				Males			
NN8	8	21	12	345.3	1177	3.41	1.00
RR8	8	30	25	344.5	2556	7.41	2.17
RR4	7	12	14	343.8	1395	4.06	1.19
RN4	8	16	14	346.9	1392	4.01	1.18
				Females			
NN8	8	39	12	230.5	964	4.18	1.00
RR8	8	26	28	230.2	2490	10.82	2.59
RR4	7	17	19	231.4	1591	6.88	1.65
RN4	8	15	19	231.9	1592	6.86	1.64

duced in progeny of female rats of the Sprague-Dawley strain when the maternal diet was restricted to 50% of ad libitum intake during gestation and lactation. These effects were similar to those previously reported in rats of the McCollum strain.

The same permanent effects, but to a less marked extent, were produced in progeny by maternal dietary restriction during gestation alone. This was demonstrated by fostering procedures in which litters from diet-restricted mothers were separated at birth into two groups of four pups each. One group of four was left with their natural mother and the other four littermates were transferred to a normally fed foster mother. Progeny in both groups grew at similar rates which were significantly below normal and consumed feed at equal rates. These results showed that normal dams and diet-restricted dams had nearly equal potential to supply nutriture to their sucklings under the reduced demands of four pups in their litter. Therefore, one plausible conclusion is that maternal dietary restriction during gestation exerts a definite influence on the development of progeny.

Male and female progeny from restricted dams required more time and larger total feed intakes to reach body weights easily attained by young normal rats during their initial 12-week active growing phase.

ACKNOWLEDGMENTS

We thank Major J. E. Del Favero, VC, U.S. Army, and Professor T. H. Yang for professional advice in animal care, and J. J. Chang and K. J. Lin for professional assistance in the study. Lucy Chang, Maggie Huang, Ruth Jean, Linda Ting, Chun-Chu Hwa, Ling Yen and Mei-Huei Chen provided technical assistance.

LITERATURE CITED

1. Chow, B. F., and C. J. Lee 1964 Effect of dietary restriction of pregnant rats on body weight gain of the offspring. J. Nutr., *82:* 10.
2. Chow, B. F. 1964 Growth of rats from normal dams restricted in diet in previous pregnancies. J. Nutr., *83:* 289.
3. Lee, C. J., and B. F. Chow 1965 Protein metabolism in the offspring of underfed mother rats. J. Nutr., *87:* 439.
4. Hsueh, A. M., C. E. Agustin and B. F. Chow 1967 Growth of young rats after differential manipulation of maternal diet. J. Nutr., *91:* 195.
5. Heggeness, F. W., D. Bindschadler, J. Chadwick, P. Conklin, S. Hulnick and M. Oaks 1961 Weight gains of overnourished and undernourished preweanling rats. J. Nutr., *75:* 39.
6. McCance, R. A., and E. M. Widdowson 1962 Nutrition and growth. Proc. Roy. Soc. (London) Series B, *156:* 326.
7. Winick, M., and A. Noble 1967 Cellular response with increased feeding in neonatal rats. J. Nutr., *91:* 179.

Performance of Rats Alternately Fed Diets Higher and Lower in Energy or Protein [1]

KARL M. BARTH AND JAMES C. McCONNELL [2]
University of Tennessee, Knoxville, Tennessee

ABSTRACT Effects of alternating higher and lower energy or protein diets on carcass composition, growth, voluntary feed intake and feed conversion were determined with male albino rats. The five dietary regimens, each fed to six singly caged rats for 32 days, were as follows: a 10% corn oil diet alternated with a 5% corn oil diet 1) every 2 days and 2) every 4 days; a 20% casein diet alternated with a 10% casein diet 3) every 2 days and 4) every 4 days; and 5) the control diet containing 7.5% corn oil and 15% casein fed throughout the trial. When rats were fed by the two fluctuating energy regimens, they consumed less feed, gained less and were less efficient in feed conversion than control rats; none of these differences was statistically significant. There were no significant differences in body moisture, protein or ether extract content of the carcasses. However, alternating dietary protein levels significantly decreased feed consumption and body weight gains ($P < 0.05$) and significantly increased carcass moisture content in the 2-day regimen as compared with control rats. Rats on the 4-day fluctuating protein regimen had a 10% increase in carcass fat.

A change in feeding regimen without an alteration in ration composition sometimes affects animal performance. The fat content of carcasses was increased considerably when rats were force-fed twice a day (termed meal eating) the same amount of feed that was consumed by control animals in many small meals (termed nibbling) throughout the whole day (1). No differences in any of the carcass measurements were observed between meal eating and nibbling swine fed on a low level of nutrition (2); in contrast, meal eating swine on a higher level of energy intake (3) and chickens (4) had leaner carcasses than nibblers. In all of these experiments, the amount of daily diet intake of the meal eaters and the nibblers was the same.

In a more drastic alteration of the feeding regimen, performance of continuously underfed rats was compared with that of rats alternately starved for 2 days and fed for 2 days (5); although total feed intake and weight gains in both groups were the same, carcasses of the intermittently starved group contained a higher percentage of fat. When ration intake of rats was alternated between ad libitum and amounts sufficient to maintain body weight (6), they consumed less feed but had a higher carcass fat content after 60 and 120 days than rats continuously fed ad libitum.

Our interest in the study of body composition arose from problems in feeding garbage to swine. Day-to-day variation was observed in garbage type and proportion of ingredients and, therefore, in its nutrient content. However, extreme fluctuations of nutrient content occurred only rarely (7), the most variable being fat, crude fiber and calcium (8, 9). Since this variation in nutrient content is reflected in the nutrients ingested by the pigs, their nutritional regimen may be compared with that of humans. This regimen, however, is in contrast to conventional methods in which relatively constant nutrient intake by most other farm animals exists over long periods.

The purpose of the present study was to simulate with rats the fluctuating nutrient intake of swine fed garbage. This was done by alternately feeding different semipurified diets for observed effects of this nutritional regimen on body weight in-

Received for publication June 21, 1968.

[1] Published with the permission of the Director of the University of Tennessee Agricultural Experiment Station, Knoxville, Tennessee.

[2] Department of Animal Husbandry — Veterinary Science.

TABLE 1

Composition of rations (air dry basis)

Ration constituent	Control	Energy		Protein	
		High	Low	High	Low
	%	%	%	%	%
Casein [1]	15.0	15.0	15.0	20.0	10.0
Sucrose	72.9	70.4	75.4	67.9	77.9
Corn oil [2]	7.5	10.0	5.0	7.5	7.5
Salt mix [3]	3.0	3.0	3.0	3.0	3.0
Vitamin mix [4]	1.6	1.6	1.6	1.6	1.6

[1] Vitamin-free test casein, Nutritional Biochemicals Corporation, Cleveland, O.

[2] Mazola, Corn Products Company, Argo, Ill.

[3] Salt mixture no. USP XIII, no. 2, Nutritional Biochemicals Corporation, containing (in percent) calcium biphosphate, 13.58; calcium lactate·$5H_2O$, 32.70; ferric citrate·$5H_2O$, 2.97; magnesium sulfate, 13.70; potassium phosphate (dibasic), 23.98; sodium biphosphate·$2H_2O$, 8.72; and sodium chloride, 4.35.

[4] Vitamin diet fortification mixture in dextrose, Nutritional Biochemicals Corporation, added to give the following amounts per 100 kg of diet: (in grams) vitamin A conc (200,000 units/g), 7.3; vitamin D conc (400,000 units/g), 0.4; α-tocopherol, 7.9; ascorbic acid, 71.9; inositol, 7.9; choline chloride, 119.9; menadione, 3.5; p-aminobenzoic acid, 7.9; niacin, 7.3; riboflavin, 1.5; pyridoxine·HCl, 1.5; thiamine·HCl, 1.5; and calcium pantothenate, 4.8; and (in milligrams) biotin, 31.9; folic acid, 143.8; and vitamin B_{12}, 2.1.

creases, voluntary diet intake, feed conversion and carcass composition.

EXPERIMENTAL PROCEDURE

Diets. The effects of energy and protein fluctuation were studied separately. For each of these two nutrients, two rations were calculated (table 1), one containing a higher level of the specific nutrient under investigation and the other a lower level of this nutrient. In the control ration, the level of this nutrient was intermediate between its level in the higher and lower rations. The content of nutrients not under investigation remained constant, except for sucrose, which was adjusted to offset differences in casein and corn oil.

Animals. Thirty male albino rats from a closed colony of the Sprague-Dawley strain, ranging from 50 to 54 days of age and averaging 182 g in weight, were used. They were assigned to five treatment groups of six animals each according to weight. Prior to the experiment, all rats were fed a commercial pelleted rat ration [3] ad libitum.

Feeding regimen. Four treatment groups of six rats each were used, two for the fluctuation of energy and two for the fluctuation of protein. In the first treatment group, the higher and lower diets were alternated every 2 days; in the second group, every 4 days. Fluctuation of energy was achieved by alternating a diet containing 10% corn oil with one containing 5% corn oil, while protein intake was fluctuated by alternating a diet containing 20% casein with another containing 10% casein.

The rats were housed singly in wire-bottom cages and fed their respective diets for 32 days. Feed and water were available ad libitum. Fresh feed was weighed out daily, and records of weighback were maintained. Body weights were recorded every 8 days, because this interval represented the completion of two feeding cycles for the 2-day fluctuating groups and one feeding cycle for the 4-day fluctuating groups.

Rat carcasses were finely ground in a hand-operated meat grinder and thoroughly mixed. Moisture (in vacuo), ether extract and protein ($N \times 6.25$) of the ground samples were determined according to AOAC methods (10) for meat samples. Differences between treatment means in body weight increase, voluntary diet intake, feed conversion, protein efficiency ratio and body composition were evaluated statistically by analysis of variance and the multiple range test (11).

RESULTS AND DISCUSSION

Fluctuation of energy. The performance of the rats is reported in table 2. Rats on both fluctuating energy dietary regimens (2-day and 4-day diet fluctuation) gained approximately 10% less body

[3] Labena, Ralston Purina Company, St. Louis, Mo.

TABLE 2

Growth, diet intake, feed conversion and carcass composition of rats

	Control	Fluctuating nutrients [1]			
		Energy		Protein	
		2 days	4 days	2 days	4 days
Number of rats	6	6	6	6	6
Initial body weight of rats, g	182 ± 6 [2]	182 ± 5	182 ± 5	182 ± 5	182 ± 5
Total voluntary diet intake, g	[a]471 ± 14	[ab]442 ± 9	[ab]441 ± 16	[b]429 ± 13	[b]427 ± 20
Body weight gain, g	[a]99 ± 4	[ab]88 ± 2	[ab]89 ± 5	[b]84 ± 6	[b]84 ± 4
Feed conversion (g diet intake/g gain)	4.8 ± 0.1	5.0 ± 0.1	4.9 ± 0.2	5.1 ± 0.2	5.1 ± 0.2
Protein efficiency ratio (g gain/g protein intake)	1.4 ± 0.03	—	—	1.3 ± 0.05	1.3 ± 0.05
Body composition (wet basis)					
Moisture, %	[a]62.3 ± 0.2	[a]62.0 ± 0.5	[a]62.3 ± 0.6	[b]64.0 ± 0.1	[ab]62.9 ± 0.6
Protein (N × 6.25), %	19.2 ± 0.4	19.2 ± 0.4	19.1 ± 0.4	19.9 ± 0.3	19.6 ± 0.3
Ether extract, %	[ab]11.6 ± 0.2	[b]12.4 ± 0.6	[ab]11.6 ± 0.6	[a]10.7 ± 0.3	[b]12.7 ± 0.1

[1] In 2- or 4-day fluctuations, rats were alternately fed diets high then low in energy or protein. Controls were fed a diet wtih intermediate levels of both nutrients throughout the experiment (32 days).
[2] Mean ± SE of the mean. Means in the same row preceded by different superscripts are significantly different ($P < 0.05$).

weight than the control rats fed the intermediate energy ration throughout the experiment. These differences in body-weight gains were not statistically significant, even when body weight increases were expressed as percent gain above initial weight (not reported). Feed conversion (grams feed per gram weight gain) was approximately the same for the three treatment groups, and no statistically significant differences between moisture, protein or ether-extract content of the carcasses on a wet basis were observed. Differences in carcass protein or ether-extract content on a moisture-free basis (not reported) also were nonsignificant.

Under the conditions of this experiment, growth, diet consumption, feed conversion and body composition were apparently not changed significantly when rats were fed a fluctuating energy diet. It might be postulated that energy requirements for rats between 160 and 300 g body weight also remain relatively unchanged when the day-to-day energy intake is not constant. In the light of conflicting responses to meal eating and nibbling between different animal species (1–4), inferences about the responses of another species, such as swine, probably cannot be made.

Fluctuation of protein. The performance of the rats fed two fluctuating protein diets also is reported in table 2. On both the 2-day and the 4-day fluctuating regimens, body-weight increases and voluntary diet intakes were significantly lower than those for control rats ($P < 0.05$). Feed conversion was less efficient, and protein efficiency ratios decreased in rats fed fluctuating protein levels, but differences were nonsignificant.

The appetite-depressing effect of the 10%-protein diet was evidently not sufficiently offset by the increased intake of the 20%-protein diet. Hence, the combined protein intake of rats fed the higher and lower diets was less than that of rats fed the 15%-protein diet. The more drastic deviation from a constant protein intake in the 4-day fluctuation did not produce more adverse effects on weight gain, ration intake, feed conversion or protein efficiency ratio than did the 2-day fluctuation of protein intake.

Decreased growth of rats caused by alternately feeding high and low protein diets

TABLE 3

Distribution of total diet intake per rat between the higher and lower nutrient diets

Dietary regimen [1]	Intake		
	Higher diet	Lower diet	Total
	g	g	g
Control			471
Energy-2	218 ± 5 [2]	224 ± 4	442
Energy-4	220 ± 8	221 ± 8	441
Protein-2	[a]224 ± 6	[b]205 ± 7	429
Protein-4	[a]223 ± 6	[b]204 ± 7	427

[1] In 2- or 4-day fluctuations, rats were alternately fed diets high then low in energy or protein. Controls were fed a diet with intermediate levels of both nutrients throughout the experiment (32 days).

[2] Mean ± SE of the mean. Means in the same row preceded by different superscripts are significantly different ($P < 0.05$).

also has been reported by van Dam-Bakker et al. (12). They used much more drastic differences in protein levels between the two alternating rations, equalized ration intake between the normally and alternately fed rats, and alternated rations more frequently. In contrast, growth of ruminants was usually not decreased when protein-deficient rations were supplemented with high protein feeds for intervals of up to 3 weeks as compared with daily supplementation (13, 14).

Body composition of control rats and rats on the fluctuating protein dietary regimen is also reported in table 2. Carcasses of the rats fed fluctuating protein levels were higher in moisture content than those of the controls. In the 2-day fluctuation, the increase in moisture content was statistically significant ($P < 0.05$). Differences in protein content among any of the treatment groups, however, were not significant. Percentage of ether extract from the 2-day fluctuated rats was somewhat lower than that from the control rats. This decrease and the increased moisture content probably were results of the lower feed intake of the group on the 2-day protein fluctuation regimen.

When the dietary protein level was changed every 4 days, rats consumed less feed than the controls. Carcasses from this treatment group, however, contained approximately 10% more ether extract than those from the control group. Specific causes for this increase in ether extract will require further study.

Ad libitum diet intake. Table 3 shows the total amount of the high energy and low energy diets voluntarily consumed by rats throughout the experiment. On both the 2-day and 4-day energy fluctuation regimens, rats consumed slightly more of the lower energy diet, probably due to an attempt to equalize energy intake from the lower energy diet. When the dietary protein content was fluctuated, however, rats consumed significantly ($P < 0.05$) more of the high protein diet on both the 2-day and the 4-day fluctuating regimens.

LITERATURE CITED

1. Cohn, C., and D. Joseph 1959 Changes in body composition attendant on force-feeding. Amer. J. Physiol., *196:* 965.
2. Friend, D. W., and H. M. Cunningham 1964 Effects of feeding frequency on metabolism, rate and efficiency of gain and on carcass quality of pigs. J. Nutr., *83:* 251.
3. Friend, D. W., and H. M. Cunningham 1967 Growth, carcass, blood and fat studies with pigs fed once or five times daily. J. Anim. Sci., 26: 316.
4. Feigenbaum, A. S., H. Fisher and H. S. Weiss 1962 Effects of "meal eating" versus "nibbling" on body composition and digestive organ weight of normal and cropectomized chickens. Amer. J. Clin. Nutr., *11:* 312.
5. Fabry, P., R. Petrasek, E. Horakova, E. Konopasek and T. Braun 1963 Energy metabolism and growth in rats adapted to intermittent starvation. Brit. J. Nutr., *17:* 295.
6. Heggeness, F. W. 1965 Effect of intermittent food restriction on growth, food utilization and body composition of the rat. J. Nutr., *86:* 265.
7. Engel, R. W., C. C. Brooks, C. Y. Kramer, D. F. Watson and W. B. Bell 1957 The composition and feeding value of cooked garbage for swine. Virginia Agr. Exp. Sta. Tech. Bull., 133.
8. Kornegay, E. T., G. W. Vander Noot, K. M. Barth, W. S. MacGrath, J. G. Welch and E. D. Purkhiser 1965 Nutritive value of garbage as a feed for swine. I. Chemical composition, digestibility and nitrogen utilization of various types of garbage. J. Anim. Sci., *24:* 319.

9. Barth, K. M., G. W. Vander Noot, W. S. MacGrath and E. T. Kornegay 1966 Nutritive value of garbage as a feed for swine. II. Mineral content and supplementation. J. Anim. Sci., *25:* 52.
10. Association of Official Agriculture Chemists 1965 Official Methods of Analysis, ed. 10. Washington, D. C.
11. Ducan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics *11:* 1.
12. Van Dam-Bakker, A. W. I., A. P. de Groot and R. Luyhen 1958 The influence of alternate high-protein and low-protein feeding on growth and reproduction and on regeneration of haemoglobin in rats. Brit. J. Nutr., *12:* 259.
13. Pope, L. S., A. B. Nelson and W. D. Campbell 1963 Feeding protein supplements to range cows at 2-, 4- or 6-day intervals. Okla. Agr. Exp. Sta. Misc. Publication, *70:* 49.
14. Kromann, R. P., E. E. Ray and A. B. Nelson 1966 Influence of frequency and source of protein supplementation on energy metabolism in feeder lambs. J. Anim. Sci., *25:* 1040.

Fluoride Toxicity in the Mouse [1]

C. W. WEBER AND B. L. REID
Poultry Science Department, University of Arizona, Tucson, Arizona

ABSTRACT Young, growing mice were fed diets containing six different levels of added fluoride; only the two highest (1500 and 2000 ppm) had any effect on either growth or mortality. A dietary level of 1500 ppm fluoride was required to cause a statistically significant growth reduction. When a dietary level of 2000 ppm was fed, 100% mortality occurred. No alteration was found in the apparent digestion of either energy, fat or protein due to fluoride feeding. Examination of numerous bones showed citric acid levels to be lowered, while tibia bone fluoride concentrations were elevated. The tibia fluoride concentrations in mice fed either 1000 or 1500 ppm of added fluoride were 45 times those of the control mice. Liver homogenates were analyzed for enzyme activities, and both cytochrome oxidase and isocitric dehydrogenase activities were lowered by both the 1000 and 1500 ppm levels of fluoride. The cytochrome oxidase activity of heart tissue was reduced only with 1500 ppm fluoride in the diet.

Fluoride toxicity has been extensively studied in rats and chicks, but there are limited data available for mice. Leone et al. (1) reported that 46 mg fluoride/kg body weight (as NaF) orally or 23 mg/kg intravenously, produced acute toxicity in mice. Segredo et al. (2) using stannous fluoride administered as a 10% solution via stomach tube, reported that the LD_{50}/24 hours was 3.54 mg in mice. These workers (2) observed no changes at autopsy or upon histologic examination which could be considered pathognomonic; and although the kidneys showed marked hyperemia, no tubular changes could be associated with chronic sodium fluoride poisoning. Segawa (3) reported that the toxic symptoms of sodium fluoride in mice were excitation and convulsions. The use of larger doses caused severe convulsions followed by tonic paralysis. It was assumed that these effects of sodium fluoride were due to activation of cell membranes by sodium fluoride itself or indicated through an anticholinesterase action. Fleming and Greenfield (4) reported that the oral or parenteral administration of sodium fluoride or calcium fluoride to pregnant mice caused changes in the structure of the jaws and teeth of neonatal mice. The changes were retardation of calcification in the jaw bones and enamel matrix plus alteration in the cell structure of the ameloblasts. Calcium fluoride seemed to be more toxic to the fetus than sodium fluoride. The fluoride was given in the drinking water at ingestion rates of 0.060 to 0.80 mg/day, and injections were at a level of 0.10 mg/day. Levels which could be tolerated without causing fetal resorption or stillbirths were approximately 0.60 to 0.70 mg calcium fluoride and 1.0 to 1.2 mg sodium fluoride.

In view of the lack of information concerning the toxic effects of fluoride in mice, the present studies were undertaken to evaluate the effects of dietary fluoride on growth, digestion and on selected enzyme activities.

MATERIALS AND METHODS

Weanling male and female mice [2] weighing 8 g were housed in raised wire screen cages at a room temperature of 26 ± 2°. Feed and water were supplied ad libitum.

In the first experiment, 11 mice/dietary treatment were selected randomly without regard to sex and housed two per cage. The second and third experimental designs consisted of 24 mice/dietary treatment using an equal number of male and female mice housed two per cage.

The dietary treatments included a basal diet (table 1) plus various levels of added sodium fluoride. In the three experiments, the following amounts of fluoride were added: 1) 0, 225 and 450 ppm; 2) 0, 450 and 900 ppm; and 3) 0, 1000, 1500 and 2000 ppm. The basal diet contained 1.35

Received for publication June 29, 1968.

[1] Arizona Agricultural Experiment Station, Journal Article no. 1364.

[2] ANSCO Laboratories, Napa, California.

TABLE 1

Composition of mouse diets

	% of diet
Soybean protein	28.00
Corn oil	6.00
Sucrose	56.96
Vitamin mixture [1]	0.14
Salt mixture [2]	8.00
Choline chloride	0.20
DL-Methionine	0.40
Cr_2O_3	0.30
Total	100.00

[1] Supplied the following per kilogram of diet: (in milligrams) ascorbic acid, 12.5; thiamine·HCl, 12.5; niacin, 100.0; riboflavin, 20.0; pyridoxine·HCl, 12.5; *d*-biotin, 1.25; calcium D-pantothenate, 75.0; vitamin B_{12}, 1.00; folic acid, 4.00; *d*-α-tocopheryl acetate, 75.0; menadione (2-methyl-1,4-naphthoquinone), 1.25; ethoxyquin, 500.0; *i*-inositol, 500.0; *p*-aminobenzoic acid, 25.0; and oxytetracycline, 25.0; and (in IU): vitamin A, 7,000; and vitamin D_2, 1,500.

[2] Supplied the following: (as percent of diet) $Ca(OH)_2$, 0.8200; KH_2PO_4, 1.9760; NaCl, 0.5000; $MnSO_4 \cdot H_2O$, 0.0336; $FeSO_4 \cdot 7H_2O$, 0.1320; $ZnSO_7$, 0.0240; $CuSO_4 \cdot 8H_2O$, 0.0020; KI, 0.0052; $CoCl_2 \cdot 6H_2O$, 0.0100; KCl, 0.5000; $MgSO_4 \cdot 7H_2O$, 0.5800; $H_2MoO_4 \cdot H_2O$, 0.003; and KBr, 0.0007.

ppm fluoride. The mice were weighed and killed at the end of the third experimental week. At that time samples of liver, kidney and heart were frozen immediately in a dry ice-acetone solution and stored at − 10° until analyzed for fatty acids and enzyme activities.

Feed and fecal samples were collected during the last 2 weeks of the experiment for chromic oxide, fat, nitrogen and bomb calorimeter analyses (5). These values were used to calculate the percentage of apparent fat digestion and digestible energy values of the diet.

Fatty acids were determined on pooled samples of liver from each treatment by the method of Metcalfe and Schmitz (6). Two humeri from each of 24 mice/dietary treatment were analyzed for bone citric acid (7). In addition two tibiae from each of 24 mice/dietary treatment were analyzed for fluoride (8). Enzyme activities were determined on the homogenized tissue samples using five males and five females per dietary treatment for succinic dehydrogenase (9), malic dehydrogenase,[3] isocitric dehydrogenase,[4] cytochrome oxidase (10) and NADH cytochrome c reductase (11). The data were analyzed by analysis of variance and Duncan's multiple range test (12).

RESULTS

Mice fed diets high in fluoride showed a relatively high tolerance to it in comparison with rats. The week 3 body weights for the first two experiments indicated no significant differences with as much as 900 ppm added fluoride (table 2). In experiment 3 no body weight differences occurred until 1500 ppm added fluoride were fed. This dietary level of fluoride caused a marked reduction in body weight gains. The feeding of 2000 ppm added fluoride produced 100% mortality, whereas the feeding of 1500 ppm added fluoride produced a 17% mortality (table 2). No other deaths occurred.

The effect of fluoride on the apparent digestion and absorption of the dietary nutrients was studied in both experiments 2 and 3 (table 3). No marked changes in apparent fat absorption or in digestible energy were noted with the feeding of sodium fluoride. Apparent digestible energy values ranged from 80 to 85%, whereas the values for apparent fat absorption ranged from 91 to 97%. Nitrogen absorption, measured only in the third experiment, was virtually unchanged, with only a 1% difference among all dietary treatments.

Further examination of fat metabolism was undertaken through analysis of liver fatty acid composition (table 4). The re-

TABLE 2

Effect of fluoride on body weights and mortality

Exp. no.	Dietary fluoride	Week 3 body wt [1]	Mortality
	ppm	*g*	%
1	0	22.1 [a]	0
1	225	23.6 [a]	0
1	450	22.7 [a]	0
2	0	22.4 [a]	0
2	450	21.4 [a]	0
2	900	20.0 [a]	0
3	0	21.2 [a]	0
3	1000	19.8 [a]	0
3	1500	14.4 [b]	17
3	2000	0 [2]	100

[1] Means not having common letter superscripts are significantly different ($P \leq 0.05$).

[2] No mice survived after the second week.

[3] Miscellaneous enzyme determination in serum. Sigma Chemical Company, St. Louis, Mo. Tech. Bull. no. 340, 1957.

[4] Isocitric dehydrogenase. Sigma Chemical Company, St. Louis, Mo. Tech. Bull. no. 175, 1961.

TABLE 3

Effect of fluoride on digestion and absorption of the dietary ingredients

Exp. no.	Dietary fluoride	Apparent digestible energy	Apparent fat absorption	Apparent nitrogen absorption
	ppm	%	%	%
2	0	80.60	91.05	—
2	450	82.48	91.73	—
2	900	84.71	92.54	—
3	0	82.39	95.57	78.06
3	1000	80.44	93.72	76.89
3	1500	81.71	96.64	77.50

TABLE 4

Effect of dietary fluoride on the fatty acid composition of livers (Exp. 2)

Fatty acid	Dietary levels of added fluoride		
	0 ppm	250 ppm	450 ppm
		% of total fat	
C_{14}	0.4	0.3	0.3
C_{16}	22.6	21.8	21.2
$C_{16:1}$	4.9	4.2	3.4
C_{18}	7.5	6.5	10.4
$C_{18:1}$	44.1	44.7	35.2
$C_{18:2}$	13.2	12.3	14.0
$C_{20:4}$	7.3	9.2	15.5
S:U [1]	0.44:1	0.42:1	0.47:1

[1] S:U = Ratio of saturated to unsaturated (including oleic) fatty acids.

sults obtained suggested a possible alteration in fat metabolism; however, statistical evaluation of the differences was not possible because pool samples were used for analysis. Liver oleic acid values decreased from 44 to 35.2% of total liver lipids with the feeding of 450 ppm fluoride. A linear increase in arachidonic acid ($C_{20:4}$) was obtained with the respective fluoride treatments (table 4). However, no marked changes in saturated-to-unsaturated fatty acid ratios (includes oleic acid) were noted.

Bone citric acid in mouse humeri showed no significant alteration with added fluoride intakes as high as 900 ppm; however, bone citric acid was reduced significantly ($P \leqq 0.05$) for mice fed either 1000 or 1500 ppm added fluoride (table 5). Values of 4.76 μg/mg bone for the controls versus 3.54 μg/mg or lower for the mice fed 1000 ppm added fluoride were observed. An analysis of bone fluoride was made for the mice in experiment 3. The controls had values of 0.15 mg/g tibia; the mice fed 1000 ppm added fluoride, 6.65 mg/g; and those fed 1500 ppm fluoride, 7.05 mg/g. These results agree with the work of Zipkin et al. (13) who reported decreased bone citric acid levels in rats with increased deposition of fluoride in bone.

Enzyme activities were measured in soft tissues of mice fed 0, 1000 and 1500 ppm of added fluoride. Liver samples showed a significant decrease ($P \leqq 0.05$) in activity for both cytochrome oxidase and isocitric dehydrogenase with high fluoride intake; only cytochrome oxidase activities were reduced in heart tissue samples (table 6). In kidney samples also, a reduced activity for isocitric dehydrogenase occurred. Activity of additional enzyme systems measured in heart, kidney and liver samples was not significantly altered (table 6).

DISCUSSION

The studies reported were initiated in an attempt to characterize the effects of toxic levels of fluoride in mice. Mice, although of the same genus, *Mus*, as rats, had a twofold tolerance to dietary fluoride in comparison with rats. Rats are usually affected by approximately 500 ppm fluoride in the diet, whereas in these studies mice showed a reduction in growth with between 1000 and 1500 ppm of added dietary fluoride. The restriction in body weight, brought about by the consumption of high dietary levels of fluoride, was evidently not caused by a gross alteration in the digestion and absorption of dietary nutrients. The data of Suttie and Phillips (14) indicated that

TABLE 5

Effect of fluoride on bone citric acid and fluoride levels

Exp. no.	Dietary fluoride	Bone citric acid [1]	Bone fluoride
	ppm	*μg/mg*	*mg/g*
1	0	4.523 [a]	—
1	225	4.346 [a]	—
1	450	4.745 [a]	—
2	0	4.812 [a]	—
2	450	4.760 [a]	—
2	900	4.992 [a]	—
3	0	4.758 [a]	0.15 [a]
3	1000	3.539 [b]	6.65 [b]
3	1500	3.206 [b]	7.05 [b]
3	2000	—	—

[1] Means not having common letter superscripts are significantly different ($P \leqq 0.05$).

TABLE 6

Effect of fluoride on tissue enzyme systems (Exp. 3)

Enzyme system	Tissue	Dietary levels of added fluoride		
		0 ppm	1000 ppm	1500 ppm
		Δ_{OD}/*minute per gram protein* [1]		
Succinic dehydrogenase	Liver	26.44 [a,2]	24.93 [a]	28.60 [a]
	Heart	31.02 [a]	13.17 [a]	64.25 [a]
Cytochrome oxidase	Liver	126.93 [a]	87.04 [b]	86.36 [b]
	Heart	67.42 [a]	55.53 [ab]	25.61 [b]
Isocitric dehydrogenase	Liver	25.41 [a]	19.03 [b]	19.15 [b]
	Kidney	87.12 [a]	75.57 [a]	70.57 [a]
	Heart	17.43 [a]	13.34 [a]	19.80 [a]
Malic dehydrogenase	Liver	140.56 [a]	154.48 [a]	155.40 [a]
	Kidney	369.54 [a]	362.54 [a]	318.43 [a]
	Heart	275.16 [a]	248.61 [a]	271.08 [a]
NADH cytochrome c reductase	Liver	24.38 [a]	26.02 [a]	27.66 [a]
	Heart	15.37 [a]	9.01 [a]	8.08 [a]

[1] Enzyme activity calculated as change in optical density per minute per gram protein.
[2] Means not having common letter superscripts are significantly different ($P \leq 0.05$).

high levels of dietary sodium fluoride caused a reduction in fat absorption in rats; this could not be confirmed using mice in the present experiments. However, our results would indicate some alteration in liver fat metabolism. Mice fed 450 ppm added fluoride had altered liver fatty acid ratios in comparison with the controls.

Increased urinary excretion and deposition of fluoride into skeletal tissues provide two mechanisms for increased fluoride tolerance. In rats the deposition of fluoride in the skeletal system occurs rapidly at first, but then proceeds at a diminishing rate up to the point of saturation (15). A flooding of soft tissues then occurs when the bones have reached 35 to 45 times the normal concentration of fluoride. The results of tibial fluoride analyses (table 5) indicated no significant increase in fluoride concentration above the 1000-ppm added dietary fluoride level. The bone fluoride levels for the 1000- and 1500-ppm treatments were approximately 45 times the controls. In addition, bone citric acid levels were lower in bones with elevated bone fluoride levels, as also demonstrated by Zipkin et al. (13).

The possible saturation of the skeletal system and overflow of fluoride into the soft tissues prompted the examination of the soft tissues for enzymatic changes. Several studies were carried out in vitro using mouse tissues to determine the effect of fluoride ions on enzyme activity estimations.[5] The following enzyme systems were either inhibited or stimulated at fluoride levels of 0.05 to 0.1 M: succinic dehydrogenase, cytochrome oxidase, isocitric dehydrogenase and NADH cytochrome c reductase.

The possibility of alterations of enzyme activities as a result of reductions in feed intakes was not studied. Feed consumption was reduced. Mice fed the control diet consumed 1 g/week more than those fed the 1000-ppm diet and 4 g/week more than those fed the 1500-ppm added fluoride diet. These differences in feed consumption could have been a factor. Starvation, type of diet or even feed restriction may cause changes in enzyme activities (16).

Morphological examinations of mice fed fluoride showed no distinguishable differences between dietary treatment or sexes which could be attributed to the dietary fluoride treatments.

Although there were differences in growth rate between the male and female mice fed the respective experimental diets, no sex-related differences in the responses were obtained as a result of the fluoride feeding. Therefore, results obtained with males and females in these studies were combined.

The ability of the mouse to tolerate larger quantities of fluoride could possibly

[5] Weber, C. W., and B. L. Reid, unpublished data. University of Arizona, 1967.

be credited to either a lower intestinal absorption or a more effective elimination of fluoride in comparison with the rat, as has been postulated for the hen by Haman et al. (17).

ACKNOWLEDGMENTS

The authors thank Mrs. Natalie Carlton for assistance in care of the animals and Mrs. Anne Nelson and Mrs. Alice Papcun for assistance in laboratory analyses.

LITERATURE CITED

1. Leone, N. C., E. F. Geever and N. C. Moran 1956 Acute and subacute toxicity studies of sodium fluoride in animals. Public Health Rep., *71:* 459.
2. Segredo, V. A., R. A. Yeary, R. Brooks and N. O. Harris 1961 Toxicity study of stannous fluoride in Swiss strain mice. J. Dent. Res., *40:* 623.
3. Segawa, T. 1955 Effect of fluoride on rabbit heart. Jap. J. Pharm. Chem., *27:* 37.
4. Fleming, H. S., and V. S. Greenfield 1954 Changes in the teeth and jaws of neonatal Webster mice after administration of NaF and CaF to the female parent during gestation. J. Dent. Res., *33:* 780.
5. Weber, C. W., and B. L. Reid 1967 Protein requirement of *Coturnix* quail to five weeks of age. Poultry Sci., *46:* 1190.
6. Metcalfe, L. D., and A. A. Schmitz 1961 The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem., *33:* 363.
7. Hess, W. C., and A. A. White 1955 A simplified method for the determination of citric acid in dentin and bone. J. Dent. Res., *34:* 462.
8. Singer, L., and W. D. Armstrong 1968 Determination of fluoride in bone with the fluoride electrode. Anal. Chem., *40:* 613.
9. Cooperstein, S. J., A. Lazarow and N. J. Kurfess 1950 A microspectrophotometric method for the determination of succinic dehydrogenase. J. Biol. Chem., *186:* 129.
10. Cooperstein, S. J., and A. Lazarow 1951 A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem., *189:* 665.
11. De Bernard, B. 1957 Studies on the terminal electron transport system. V. Extraction of a soluble DPNH cytochrome C reductase from the electron transport particle. Biochim. Biophys. Acta, *23:* 510.
12. Duncan, D. B. 1955 The new multiple range and F test. Biometrics, *11:* 1.
13. Zipkin, I., R. Schraer, H. Schraer and W. A. Lec 1963 Influence of fluoride on the citrate content of the bones of growing rats. Arch. Oral Biol., *8:* 119.
14. Suttie, J. W., and P. H. Phillips 1960 Fat utilization in the fluoride-fed rat. J. Nutr., *72:* 429.
15. Stookey, G. K., D. B. Crane and J. C. Muhler 1963 Role of skeleton and kidney in fluoride absorption in rat. Proc. Soc. Exp. Biol. Med., *113:* 366.
16. Knox, W. E., and O. Greengard 1965 The regulation of some enzymes of nitrogen metabolism. In: An Introduction to Enzyme Physiology, G. Weber, ed., Pergamon Press, New York, pp. 247–313.
17. Haman, K., P. H. Phillips and J. G. Halpin 1936 The distribution and storage of fluorine in the tissue of laying hens. Poultry Sci., *15:* 154.

Effect of Dietary Protein Source and Corn Oil and Cellulose Levels on Strontium-calcium Discrimination in Growing Rats [1,2]

E. W. HARTSOOK, R. L. COWAN, P. T. CHANDLER [3] AND J. B. WHELAN [4]
Department of Animal Science, The Pennsylvania State University, University Park, Pennsylvania

ABSTRACT The effect of diet composition on strontium-calcium discrimination in the growing rat was investigated using tracer doses of ^{89}Sr and ^{45}Ca administered by stomach tube to both sexes in a 2 × 2 × 3 factorial experiment whose variables in addition to sex (S) were protein (P), casein or zein at a 20% dietary level, cellulose (C), at a 3 or 12% dietary level, and corn oil (CO), at a zero, 4 or 8% dietary level. Criteria of response were the 2-hour disappearance of each isotope from the gastrointestinal tract, content of each isotope in the femurs and blood serum (expressed as percentage of administered dose) and the following $^{89}Sr/^{45}Ca$ ratios; that absorbed from the gastrointestinal tract, that contained in femurs and that contained in blood serum. Significant main effects of treatments were: casein produced 41 and 42% greater absorption and 48 and 59% greater femur content of ^{89}Sr and ^{45}Ca, respectively, than zein, but did not alter either $^{89}Sr/^{45}Ca$ absorbed or $^{89}Sr/^{45}Ca$ in femurs. Casein produced a 69% reduction of serum ^{45}Ca as compared with zein which caused the $^{89}Sr/^{45}Ca$ in serum to be 534% greater than the value for zein. When dietary cellulose increased from 3 to 12%, ^{89}Sr of bone and ^{45}Ca of serum were decreased by 21 and 25%, respectively. Male rats had 34 and 14% smaller quantities of femur ^{89}Sr and ^{45}Ca, respectively, than females. The main effect of dietary level of CO was nonsignificant. Significant interactions for respective criteria were: absorbed ^{45}Ca, C with CO; bone ^{89}Sr, C with CO; bone ^{45}Ca, C with CO; $^{89}Sr/^{45}Ca$ in femur, P with S and P with CO; serum ^{89}Sr, P with C; and serum ^{45}Ca, P with C and S with C.

Discrimination against strontium in favor of calcium has been shown to occur during the physiological processes of intestinal absorption, renal excretion, fetal uptake and milk secretion. The data concerning this phenomenon have recently been summarized by Comar (1). Much attention has been given to the factors that modify such discrimination during intestinal absorption of strontium and calcium and many substances have been found that affect the rates of absorption of these elements — lactose (2, 3), amino acids (3–5), phosphorus (6), and alginic acid (7). Recently, Marcus and Wasserman (8) found that both lactose and lysine altered strontium-calcium discrimination during intestinal absorption by changing the absorption of both alkaline earths proportionally and not by changing the comparative rate constants of strontium and calcium transport across the mucosa of the gut.

In 1963, Ryushi and Enomoto (9) studied the effect of diet composition upon strontium-calcium discrimination in the rat. These workers compared the effect of rice, milk and commercial rat diets in this regard. The experimental work was to answer the question whether the lower values of strontium-calcium observed ratios ($OR_{bone-diet}$) [5] noted in the Japanese population, as compared with that of the United Kingdom and the United States, was accounted for in part by the larger rice intake of the Japanese. These workers found the $OR_{bone-diet}$ of rats fed rice diets to be almost equal to that for milk diets and significantly greater than that for a commercial

Received for publication May 18, 1968.
[1] Authorized for publication on May 10, 1968 as paper no. 3415 in the journal series of the Pennsylvania Agricultural Experiment Station.
[2] Supported in part by AEC Research Contract no. AT(30-1)-3470.
[3] Department of Dairy Science. Present address: Department of Dairy Science, Virginia Polytechnic Institute, Blacksburg, Virginia 24060.
[4] Present address: Virginia Cooperative Wildlife Research Unit, 105 Price Hall, Virginia Polytechnic Institute, Blacksburg, Virginia 24060.
[5] The strontium-calcium observed ratio (OR) is defined by Comar et al. (10) in the following manner:

$$OR_{sample\text{-}precursor} = \frac{Sr/Ca \text{ of sample}}{Sr/Ca \text{ of precursor}}$$

diet. The milk diet was composed of 92.6% powdered whole milk and 3.7% each of dried yeast and cellulose; the rice diet was composed of 94.9% milled rice powder and 2.5% each of dried yeast and cellulose; and the commercial diet was of undisclosed composition, evidently contained varying quantities of nutrients — particularly the macronutrients, proteins, lipids, and carbohydrates. Also, wide differences probably existed in biological value of proteins (relative amino acid concentrations), qualitative and quantitative lipid composition and cellulose content. The same general comments can be made regarding the work of Comar et al. (10) in which $OR_{bone-diet}$ of rats fed condensed milk diets were found to be greater than for those fed condensed milk – ground yellow corn diets, which in turn were greater than for those fed commercial diets.

The purpose of the work reported here was to study the effect of differing protein biological values and dietary corn oil and cellulose levels on strontium-calcium discrimination in the growing rat.

EXPERIMENTAL

Ninety-six weanling rats of the Wistar strain (48 of each sex) averaging 53 g in body weight were assigned to 12 outcome groups and fed ad libitum the experimental diets, which constituted a 2 × 2 × 3 factorial experimental design. Tap water was given ad libitum.

The experimental diets contained these respective percentages of ingredients: protein (either casein or zein), 20; mineral mix (11), 2; vitamin mixture,[6] 1; cellulose,[7] either 3 or 12; corn oil, either zero, 4 or 8; and corn starch to total 100. Respective diet numbers and variables are indicated in the column headings of tables 1 and 2. The diets were group-fed to rats housed either two or three per screen-bottom cage for a 25-day period.[8] Then food was withdrawn approximately 20 hours before tracer doses of ^{89}Sr and ^{45}Ca were administered to the rats by stomach tube. The dosing solution of physiological saline contained approximately 4.3 μCi of ^{89}Sr and 6.0 μCi of ^{45}Ca per milliliter and was administered at the rate of 0.01 ml/g of live weight. Exactly 2 hours after dosing, the rats were killed by a blow on the head and the following samples were obtained: blood serum, both femurs, and the gastrointestinal tract with contents intact.

The samples were ashed at 550° and the ash taken up in dilute hydrochloric acid (1 HCl–3H_2O, v/v), then made to volume and counted windowless and with an aluminum absorber (thickness = 7.3 mg/cm²) in a gas-flow counter.[9] Counting rates were corrected for self-absorption and the activity of each isotope was arrived at by utilizing the counting of ^{89}Sr and ^{45}Ca standard solutions and the dosing solution with and without the aluminum absorber. The isotope contents of the samples were expressed as percentage of administered dose. The effects of the dietary treatments upon strontium-calcium discrimination were compared by means of the ratios: $^{89}Sr/^{45}Ca$ absorbed, $^{89}Sr/^{45}Ca$ of femurs and $^{89}Sr/^{45}Ca$ of serum.

The statistical evaluation of the data was effected using the analysis of variance with calculation of sums of squares by the method of unweighted means (12). Values of P of 0.10 and below were considered to be statistically significant.

RESULTS AND DISCUSSION

In tables 1 and 2 are presented for 20% casein and 20% zein diets, respectively, data of ^{89}Sr and ^{45}Ca absorbed and of the ^{89}Sr and ^{45}Ca contents in femurs and in blood serum. Also presented are these ratios: $^{89}Sr/^{45}Ca$ absorbed, $^{89}Sr/^{45}Ca$ of femurs and $^{89}Sr/^{45}Ca$ of blood serum. Due to rounding errors, the mean values of ratios are not always exactly equal to the quotient of mean values shown for tissue contents. The number of observations was usually eight for each criterion; exceptions are duly noted. Many animals receiving zein diets did not survive until the time of

[6] Vitamin diet fortification mixture in dextrose (obtained from Nutritional Biochemicals Corporation, Cleveland) containing: (in milligrams per gram of mixture) vitamin A concentrate (crystalline vitamin A acetate, 200,000 U/g), 4.5; vitamin D concentrate (crystalline calciferol, D_2, 400,000 U/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Calcium pantothenate, 3.0; and (in micrograms per gram of mixture) biotin, 20.0; folic acid, 90.0; and vitamin B_{12}, 1.35.

[7] Alphacel, Nutritional Biochemicals Corporation.

[8] During the last 5 days of the period, to increase survival and reduce inanition, the zein diets were supplemented with L-lysine·HCl and DL-tryptophan at rates of 4.3 and 0.4 g/kg, respectively.

[9] Nuclear-Chicago Corporation, Des Plaines, Illinois.

TABLE 1

^{89}Sr and ^{45}Ca absorbed and contained in rat blood serum and femurs 2 hours after dosing (20% casein diet)

Diet no.	1	2	3	4	5	6
Cellulose, %	3	3	3	12	12	12
Corn oil, %	0	4	8	0	4	8
No. of animals	8	7	8	8	8	7
Male	4	4	4	4	4	3
Female	4	3	4	4	4	4
^{89}Sr absorbed, % of dose	63.03 ± 19.80 [1]	69.19 ± 23.98	68.56 ± 26.15	73.47 ± 21.82	53.22 ± 12.80	58.08 ± 19.71
^{45}Ca absorbed, % of dose	80.95 ± 10.92	83.52 ± 15.03	86.07 ± 13.07	84.01 ± 22.32	78.22 ± 12.30	79.71 ± 12.18
$\frac{^{89}Sr \text{ absorbed, \% of dose}}{^{45}Ca \text{ absorbed, \% of dose}}$	0.78 ± 0.21	0.82 ± 0.20	0.77 ± 0.22	0.92 ± 0.26	0.68 ± 0.09	0.74 ± 0.28
^{89}Sr in both femurs, % of dose	4.86 ± 1.53	5.93 ± 2.76	5.31 ± 2.58 (7)	5.70 ± 3.45	3.37 ± 1.17(7)	4.74 ± 2.20
^{45}Ca in both femurs, % of dose	7.28 ± 2.34	8.16 ± 2.75	8.11 ± 2.46 (7)	9.87 ± 3.67	9.09 ± 2.23(7)	7.66 ± 2.92
$\frac{^{89}Sr \text{ in both femurs, \% of dose}}{^{45}Ca \text{ in both femurs, \% of dose}}$	0.72 ± 0.28	0.70 ± 0.15	0.61 ± 0.25 (7)	0.60 ± 0.28	0.42 ± 0.04(7)	0.64 ± 0.22
^{89}Sr in serum, % of dose in 0.5 ml	0.070 ± 0.028(6) [2]	0.071 ± 0.012(4)	0.085 ± 0.052(7)	0.089 ± 0.018(7)	0.090 ± 0.023	0.089 ± 0.056(5)
^{45}Ca in serum, % of dose in 0.5 ml	0.006 ± 0.002(6)	0.007 ± 0.003(4)	0.004 ± 0.004(7)	0.010 ± 0.008(7)	0.004 ± 0.002	0.009 ± 0.008(5)
$\frac{^{89}Sr \text{ in serum, \% of dose in 0.5 ml}}{^{45}Ca \text{ in serum, \% of dose in 0.5 ml}}$	16.02 ± 11.69 (6)	10.52 ± 3.68 (4)	33.65 ± 46.63 (7)	13.76 ± 10.18 (7)	32.52 ± 25.60	12.39 ± 5.81 (5)

[1] Mean ± SD (SD of individuals in the group).

[2] Number of observations when different from number in column heading.

TABLE 2

^{89}Sr and ^{45}Ca absorbed and contained in rat blood serum and femurs 2 hours after dosing (20% zein diet)

Diet no.	7	8	9	10	11	12
Cellulose, %	3	3	3	12	12	12
Corn oil, %	0	4	8	0	4	8
No. of animals	5	5	8	6	7	6
Male	3	3	4	3	4	2
Female	2	2	4	3	3	4
^{89}Sr absorbed, % of dose	53.19 ±25.98 [1]	42.65 ±21.98 (4) [2]	42.42 ±28.58(6)	57.84 ±30.03	50.13 ±29.56(6)	24.16 ±13.98
^{45}Ca absorbed, % of dose	63.90 ±22.36	49.75 ±31.36 (4)	62.95 ±18.24(6)	70.53 ±21.74	61.61 ±24.64(6)	33.22 ± 9.71
$\frac{^{89}Sr \text{ absorbed, \% of dose}}{^{45}Ca \text{ absorbed, \% of dose}}$	0.84 ± 0.24	1.30 ± 1.06 (4)	0.63 ± 0.24(6)	0.78 ± 0.21	0.83 ± 0.40(6)	0.85 ± 0.63
^{89}Sr in both femurs, % of dose	2.76 ± 1.74	4.22 ± 2.31	4.12 ± 2.55	3.66 ± 1.42	3.45 ± 2.66	1.06 ± 0.70
^{45}Ca in both femurs, % of dose	4.93 ± 0.88	5.11 ± 1.80	6.41 ± 2.93	5.42 ± 1.01	5.19 ± 3.32	3.45 ± 1.51
$\frac{^{89}Sr \text{ in both femurs, \% of dose}}{^{45}Ca \text{ in both femurs, \% of dose}}$	0.54 ± 0.29	0.79 ± 0.40	0.71 ± 0.53	0.65 ± 0.18	0.82 ± 0.88	0.32 ± 0.18
^{89}Sr in serum, % of dose in 0.5 ml	0.089± 0.033(4)	0.066± 0.062(4)	0.083± 0.037	0.035± 0.023	0.066± 0.047	0.049± 0.034(5)
^{45}Ca in serum, % of dose in 0.5 ml	0.028± 0.016(4)	0.019± 0.013(4)	0.034± 0.012	0.016± 0.010	0.016± 0.007	0.019± 0.008(5)
$\frac{^{89}Sr \text{ in serum, \% of dose in 0.5 ml}}{^{45}Ca \text{ in serum, \% of dose in 0.5 ml}}$	3.48 ± 1.11 (4)	3.08 ± 1.42 (4)	2.52 ± 0.98	2.34 ± 1.07	4.18 ± 2.30	2.95 ± 2.64 (5)

[1] Mean ± SD (SD of individuals in the group).

[2] Number of observations when different from number in column heading.

dosing and a few animals receiving casein diets were lost.[10] Missing values resulted from insufficient serum for analysis and inadvertent noncounting of samples with the aluminum absorber.

Values of $^{89}Sr/^{45}Ca$ absorbed observed in this work are generally within the range of values of $OR_{absorbed-dose}$[11] reported in the literature (13), and also reflect relative differences in response to the various treatments. The ratios of $^{89}Sr/^{45}Ca$ of femurs and $^{89}Sr/^{45}Ca$ of serum are not comparable with literature values of $OR_{bone-dose}$ and $OR_{serum-dose}$, respectively. This is true because the 2-hour experimental period used did not allow either a steady state of intake of the radioisotopes to develop or the renal discrimination against strontium to become effective — conditions explicit in the formulation of the OR notation (13). Nevertheless, these ratios are useful in evaluating relative differences in response to the various treatments.

The results of statistical evaluation of the data appear in tables 3 and 4. Table 3 shows the responses to the various treatments and the significance of the main effects of dietary protein source, sex and dietary levels of cellulose and corn oil. The feeding of casein significantly increased absorption of both ^{89}Sr and ^{45}Ca over that of animals receiving the zein diets. This can be explained perhaps by zein being void of lysine, a dietary factor previously shown to enhance calcium absorption (4, 5). No significant difference was found in $^{89}Sr/^{45}Ca$ absorbed for the two proteins although this criterion for both proteins (as well as the remaining dietary variables) was less than unity, indicating discrimination against strontium during intestinal absorption. Values of the "instantaneous intestinal strontium-calcium discrimination factor," as defined by Marcus and Wasserman (8), varied from 0.59 to 0.70 for the various dietary variables and averaged 0.66. This was taken as being in general agreement with the average value of 0.72 reported by the cited workers and their finding that this criterion tended to vary in response to changes in dietary composition.

Without exception, for all dietary variables and both sexes, numerically larger percentages of the administered dose of ^{45}Ca than of ^{89}Sr appeared in the bone, and values of $^{89}Sr/^{45}Ca$ of femurs were less than unity. Rats receiving casein diets deposited significantly higher percentages of ^{89}Sr and ^{45}Ca in femurs than those receiving zein diets. An important factor operative here is the essentially normal growth of the animals fed casein and the inanition and poor growth of the animals fed zein. These factors in the latter animals would reduce the amount of metabolically active bone to serve as uptake sites. Males deposited significantly less ^{89}Sr in the femurs, but significantly more ^{45}Ca, than females. As a consequence of this situation, the males exhibited a significantly lower value of $^{89}Sr/^{45}Ca$ of femurs compared with females. Varying the dietary cellulose level produced a significant difference in bone ^{89}Sr uptake with the lesser uptake in animals receiving the 12% (high) cellulose diets. This observation and the trend toward lower values for $^{89}Sr/^{45}Ca$ of femurs with the high cellulose level can be viewed as in agreement with the findings of Ryushi and Enomoto (9) and Comar et al. (10) reporting in longer-term feeding experiments that commercial rat diets (usually containing approximately 6% of crude fiber) showed lower values of $OR_{bone-diet}$ than milk diets having lower percentages of crude fiber.

The content of ^{89}Sr and ^{45}Ca in blood serum at the conclusion of a 2-hour experiment is the resultant of absorption from the gastrointestinal tract, deposition in bone and other tissues and excretion in the urine. Without exception, for all dietary variables and both sexes, numerically larger percentages of dose for ^{89}Sr than ^{45}Ca were found in serum and values of $^{89}Sr/^{45}Ca$ of serum were much greater than unity, averaging 11.2. Casein produced significantly lower serum ^{45}Ca values than zein which resulted in significantly larger values of $^{89}Sr/^{45}Ca$ of serum for casein diets. The 3% cellulose diets elicited significantly larger serum ^{45}Ca values than the 12% cellulose diets.

[10] In the initial 20 days of the experiment, the animals were used in a student laboratory in connection with a course in animal nutrition. A few animals were inadvertently lost.

[11] The Comar et al. notation (10) of $OR_{absorbed-dose}$ reduces to $\frac{\text{Sr absorbed, \% of dose}}{\text{Ca absorbed, \% of dose}}$ when Sr and Ca of the sample (i.e., absorbed) are expressed as percentage of administered dose and Sr/Ca of the precursor (i.e., the dose) is unity.

TABLE 3

Mean responses to treatments and statistical significance of main effects

Treatment	Absorbed		^{89}Sr absorbed,[1] % of dose / ^{45}Ca absorbed, % of dose	Bone		^{89}Sr in femurs,[1] % of dose / ^{45}Ca in femurs, % of dose	Blood serum		^{89}Sr in serum,[1] % of dose in 0.5 ml / ^{45}Ca in serum, % of dose in 0.5 ml
	^{89}Sr	^{45}Ca		^{89}Sr	^{45}Ca		^{89}Sr	^{45}Ca	
	% of dose			*% of dose/femurs*			*% of dose/0.5 ml ×10³*		
Casein	63.93 [c]	82.15 [d]	0.779	4.94 [c]	8.18 [d]	0.609	80.19	6.84 [d]	19.35 [d]
Zein	45.28	57.54	0.838	3.33	5.14	0.662	64.08	21.92	3.05
Male	51.72	66.79	0.835	3.30 [c]	6.16 [a]	0.513 [c]	72.92	15.27	10.44
Female	57.49	72.90	0.782	4.96	7.16	0.758	71.36	13.49	11.96
Cellulose, %									
3	56.10	71.34	0.822	4.61 [a]	6.72	0.691	75.78	16.42 [a]	11.38
12	53.12	68.35	0.794	3.65	6.60	0.580	68.50	12.33	11.02
Corn oil, %									
0	62.34	75.53	0.824	4.32	6.91	0.641	69.79	14.98	8.99
4	53.71	68.58	0.860	4.36	6.64	0.709	72.12	11.31	12.66
8	47.76	65.41	0.741	3.71	6.43	0.557	74.50	16.85	11.95

[1] Quotient.
[a] Significant at $P = 0.10$.
[b] Significant at $P = 0.05$.
[c] Significant at $P = 0.01$.
[d] Significant at $P = 0.001$.

TABLE 4

Significant interactions to treatments for the criteria of absorbed ^{45}Ca, bone ^{89}Sr and ^{45}Ca, $\frac{^{89}Sr \text{ in femurs, \% of dose}}{^{45}Ca \text{ in femurs, \% of dose}}$ and serum ^{89}Sr and ^{45}Ca

Cellulose, %	Corn oil, %			Avg, 3 corn oil levels
	0	4	8	
	A. Absorbed ^{45}Ca, % of dose [a]			
3	73.79	66.60	73.61	71.34
12	77.27	70.57	57.20	68.35
Avg, both cellulose levels	75.53	68.58	65.41	
	B. Bone ^{89}Sr, % of dose/2 femurs [c]			
3	3.97	5.24	4.63	4.61 [g]
12	4.68	3.50	2.78	3.65 [g]
Avg, both cellulose levels	4.32	4.36	3.71	
	C. Bone ^{45}Ca, % of dose/2 femurs [a]			
3	6.17	6.72	7.28	8.72
12	7.65	6.56	5.58	8.60
Avg, both cellulose levels	7.91	7.64	7.43	

Sex	Protein		Avg, both proteins
	Casein	Zein	
	D. $\frac{^{89}Sr \text{ in femur, \% of dose}}{^{45}Ca \text{ in femur, \% of dose}}$ [e]		
Male	0.590	0.435	0.513 [i]
Female	0.628	0.888	0.758 [i]
Avg, both sexes	0.609	0.662	

Corn oil, %	Protein		Avg, both proteins
	Casein	Zein	
	E. $\frac{^{89}Sr \text{ in femur, \% of dose}}{^{45}Ca \text{ in femur, \% of dose}}$ [b]		
0	0.663	0.618	0.641
4	0.556	0.861	0.709
8	0.608	0.506	0.557
Avg, 3 corn oil levels	0.609	0.662	

Cellulose, %	Protein		Avg, both proteins
	Casein	Zein	
F. Serum ^{89}Sr, % of dose/0.5 ml $\times 10^3$ [e]			
3	72.0	79.5	75.8
12	88.3	48.7	68.5
Avg, both cellulose levels	80.2	64.1	
G. Serum ^{45}Ca, % of dose/0.5 ml $\times 10^3$ [f]			
3	5.5	27.3	16.4 [g]
12	8.2	16.5	12.3 [g]
Avg, both cellulose levels	6.8 [j]	21.9 [j]	

Cellulose, %	Sex		Avg, both sexes
	Male	Female	
H. Serum ^{45}Ca, % of dose/0.5 ml $\times 10^3$ [d]			
3	19.3	13.5	16.4 [g]
12	11.2	13.5	12.3 [g]
Avg, both cellulose levels	15.3	13.5	

[a] Interaction significant at $P = 0.10$.
[b] Interaction significant at $P = 0.09$.
[c] Interaction significant at $P = 0.08$.
[d] Interaction significant at $P = 0.07$.
[e] Interaction significant at $P = 0.05$.
[f] Interaction significant at $P = 0.01$.
[g] Main effect significant at $P = 0.10$.
[h] Main effect significant at $P = 0.05$.
[i] Main effect significant at $P = 0.01$.
[j] Main effect significant at $P = 0.001$.

Table 4 depicts mean values for criteria of response for which significant first order interactions were observed. All remaining first-order interactions were not significant. All interactions of order higher than first were combined in analyses of variance for each criterion of response — none of these was statistically significant. The significant first-order interactions are presented as subsections of table 4. Subsection A of table 4 showed that there is a general tendency for absorbed ^{45}Ca to decrease as corn oil percentage increased, but the opposite tendency when cellulose level increased. At the 3% cellulose level, however, a lower value of absorbed ^{45}Ca resulted at the 4% corn oil level than at the zero and 8% levels. No explanation for this observation is at hand. Nor is an explanation apparent for the exception to increased ^{45}Ca absorption that occurred when 8% corn oil was fed. In subsections B and C of table 4 data are presented describing the significant interaction of cellulose level with corn oil level on the criteria of bone ^{89}Sr and bone ^{45}Ca, respectively. For each criterion at the 12% cellulose level there was a decrease in bone content as the corn oil percentage was increased. At the 3% cellulose level, bone ^{45}Ca increased as the corn oil percentage increased, whereas bone ^{89}Sr increased as the corn oil percentage increased to 4% and then decreased as corn oil was further increased to 8%. Subsections D and E of table 4 include data concerning, respectively, the interaction of protein with sex of the animal and with percentage of dietary corn oil for the criterion $^{89}Sr/^{45}Ca$ of femurs. Of course, the main effect of sex of animal resides in the significantly greater $^{89}Sr/^{45}Ca$ of femurs (lesser discrimination against strontium) in the female than in the male. Males receiving the zein diet, however, exhibit a lower $^{89}Sr/^{45}Ca$ of femurs (greater discrimination against strontium) than males fed casein, whereas in females the reverse situation prevails. Apparent curvilinearity is evident in the data for $^{89}Sr/^{45}Ca$ of femurs for each protein source as the corn oil percentage is increased. The data of subsections A, B, C, D and E of table 4 can become the basis for further research whose primary emphasis would involve energy metabolism. The principal differences between diets containing two levels of cellulose and three levels of corn oil are, respectively, level of metabolizable energy and the ratio of fat to carbohydrate calories. The existence of significant interactions between protein source and sex of animal, and between protein source and corn oil percentage, also indicate involvement of energy metabolism, since varying quantities of lipids, carbohydrates and amino acids and their metabolites would be present in biological systems which vary qualitatively and quantitatively in steroid hormones. Subsections F, G and H of table 4 may be considered together. Subsections F and G, respectively, concern the interactions of protein source and cellulose percentage upon the criteria of serum ^{89}Sr and ^{45}Ca. Casein diets containing 3% cellulose show a lesser value for serum ^{89}Sr than those containing 12% cellulose. The inverse was observed for zein diets — 3% cellulose produced higher values of serum ^{89}Sr than 12% cellulose. Parallel changes in serum ^{45}Ca were observed, but in each instance the percentage of dose of $^{45}Ca/0.5$ ml of serum was of lesser magnitude. Subsection H shows that serum ^{45}Ca in females did not change as dietary cellulose was increased from 3 to 12%, whereas males exhibited a significant decrease in serum ^{45}Ca with the increase in cellulose percentage.

ACKNOWLEDGMENTS

The authors thank Mrs. Pamala Briggs and Dr. K. R. Bennett, respectively, for conducting radiochemical analyses and providing guidance in statistical analyses of the data.

LITERATURE CITED

1. Comar, C. L. 1963 In: The Transfer of Calcium and Strontium Across Biological Membranes, ed., R. H. Wasserman. Academic Press, New York, p. 405.
2. Wasserman, R. H., C. L. Comar and M. M. Nold 1956 The influence of amino acids and other organic compounds on the gastrointestinal absorption of calcium^{45} and strontium^{89} in the rat. J. Nutr., 59: 371.
3. Lengemann, F. W., and C. L. Comar 1961 Distribution of absorbed strontium-85 and calcium-45 as influenced by lactose. Amer. J. Physiol., 200: 1051.
4. Wasserman, R. H., C. L. Comar, J. C. Schooley and F. W. Lengemann 1957 Interrelated effects of L-lysine and other dietary factors on the gastrointestinal absorption of

calcium 45 in the rat and chick. J. Nutr., 62: 367.
5. Raven, A. M., F. W. Lengemann and R. H. Wasserman 1960 Studies of the effect of lysine on the absorption of radiocalcium and radiostrontium by the rat. J. Nutr., 72: 29.
6. Kostial, K., A. Lutkić, N. Gruden, S. Vojvodić and G. E. Harrison 1963 The effect of dietary phosphorus on the metabolism of calcium and strontium in the rat. Int. J. Radiat. Biol., 6: 431.
7. Moore, W., Jr., and R. L. Elder 1965 Effect of alginic acid on the movement of strontium-85 and calcium-45 across surviving ileal segments. Nature, 206: 841.
8. Marcus, C. S., and R. H. Wasserman 1965 Comparison of intestinal discrimination between calcium 47, strontium 85, and barium 133. Amer. J. Physiol., 209: 973.
9. Ryushi, I., and Y. Enomoto 1963 Strontium-calcium discrimination in rats feeding on rice, milk and commercial diets. Health Phys., 9: 717.
10. Comar, C. L., R. H. Wasserman and M. M. Nold 1956 Strontium-calcium discrimination factors in the rat. Proc. Soc. Exp. Biol. Med., 92: 859.
11. Jones, J. H., and C. Foster 1942 A salt mixture for use with basal diets either low or high in phosphorus. J. Nutr., 24: 245.
12. Snedecor, G. W. 1956 Statistical Methods Applied to Experiments in Agriculture and Biology, ed. 5. The Iowa State College Press, Ames.
13. Comar, C. L., and R. H. Wasserman 1964 In: Mineral Metabolism, An Advanced Treatise, vol. 2, part A, eds., C. L. Comar and F. Bronner. Academic Press, New York, p. 523.

Copper Interference with the Intestinal Absorption of Zinc-65 by Rats

DARRELL R. VAN CAMPEN

U. S. Plant Soil and Nutrition Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Ithaca, New York

ABSTRACT Earlier studies demonstrated that high levels of zinc interfered with ^{64}Cu absorption and indicated that this interference was mediated at the intestinal level. The studies reported here were conducted to determine if the reverse were also true, that is, would copper depress ^{65}Zn absorption and, if so, what was the site of the interference? The ^{65}Zn was put into isolated duodenal segments and copper was administered either intraduodenally or intraperitoneally. When both copper and ^{65}Zn were put into the ligated segment, subsequent absorption of ^{65}Zn was depressed. When ^{65}Zn was given intraduodenally and copper was given intraperitoneally, however, no depression in the subsequent absorption of ^{65}Zn resulted. Copper concentrations in all of the sampled, nonintestinal tissues were as high or higher in the rats dosed intraperitoneally as in the rats dosed intraduodenally. This would indicate that copper did not depress ^{65}Zn uptake by first building up to some "critical" level in nonintestinal tissues and subsequently blocking zinc absorption. Rather, the results of these experiments suggest that copper interference with ^{65}Zn uptake was mediated at the intestinal level. These observations, and those reported previously, lead to the tentative conclusion that there is a mutual antagonism between zinc and copper during the absorption process and this antagonism apparently takes place either in or on the intestinal epithelium.

The reversal of certain symptoms of zinc toxicity by treatment with copper has been observed by many investigators (1–5). Despite the large amount of work done on this problem, however, the mechanism by which the zinc-copper antagonism is mediated is still poorly understood. In a recent publication (6), we reported that zinc interfered with copper absorption in rats and that this interference appeared to be relatively specific for zinc. In subsequent work (7), it was observed that zinc interfered with the absorption process if zinc was put into an isolated intestinal segment along with ^{64}Cu but had no effect if administered intraperitoneally. This suggested that the zinc effect was mediated either in or on the intestine and might be due to competition with copper. If this is the case, the reverse situation would also be expected to occur, that is, one would expect copper to impede zinc absorption. The experiments reported in this paper were designed to investigate this possibility.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain weighing between 275 and 350 g were used in these experiments. They were housed in stainless steel cages with raised wire floors and were fed a commercial pelleted ration [1] that contained 10 ppm copper and 45 ppm zinc. Rats were allocated to the various treatments in a randomized block experimental design on the basis of their body weight.

The ^{65}Zn (specific activity = 0.721 Ci/g) was received as the chloride,[2] and was diluted to a zinc concentration of 10 μg/ml with either distilled water or a cupric nitrate solution. Following an overnight fast, each rat received 0.5 ml of one of these zinc solutions containing a tracer dose (5 μg) of ^{65}Zn and either zero or 200 μg of copper. In all cases the ^{65}Zn was put into an isolated, in vivo, duodenal segment approximately 7.0 cm long. The preparation of these segments has been described previously (6, 8).

Five treatment combinations were used: 1) control treatments in which ^{65}Zn alone was given intraduodenally (ID); 2) cop-

Received for publication August 19, 1968.

[1] Big Red Dog Chow, Agway, Inc., Syracuse, N. Y. Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the U. S. Department of Agriculture.

[2] ^{65}Zn was obtained from Union Carbide Corporation, Tuxedo, N. Y.

per and ^{65}Zn were both given intraduodenally; 3) copper was given intraperitoneally at the same time that ^{65}Zn was administered intraduodenally; 4) copper was given intraperitoneally at 2 hours before the intraduodenal administration of ^{65}Zn; and 5) copper was given intraperitoneally at 18 hours before administration of ^{65}Zn.

Three hours after administration of ^{65}Zn, the rats were anesthetized; a blood sample was taken by heart puncture and the anesthetized rats were decapitated. The kidneys and the liver were removed and both kidneys and a liver sample were counted.[3] The isolated intestinal segment was removed from the animal and the segment and its contents were separated; the ^{65}Zn content of the excised segment and its contents was determined. After counting, all of the sampled tissues were wet digested with a mixture of nitric and sulfuric acids, and the concentrations of copper and zinc in each tissue were determined by atomic absorption spectrophotometry.[4]

Data were subjected to an analysis of variance and individual means were compared using a multiple range test (9). Statements of significance are based on the 5% level of probability.

RESULTS

The influence of copper on the uptake of ^{65}Zn by four tissues (blood, heart, kidneys and liver) and on the distribution of ^{65}Zn among these tissues is indicated by the data of table 1. The percentage of the dose in the four sampled tissues is one index of the amount of ^{65}Zn that was absorbed. A comparison of the control with the ID-0 treatments in table 1 indicates that when copper was put into the ligated segment along with ^{65}Zn, subsequent absorption of the ^{65}Zn was impeded. If copper was given intraperitoneally, either simultaneously with or 2 hours prior to ^{65}Zn administration, it did not significantly affect ^{65}Zn uptake. If copper was given intraperitoneally at 18 hours before the intraduodenal administration of ^{65}Zn, the subsequent absorption of ^{65}Zn from the ligated segment was significantly greater than that of the control rats. The intraduodenal administration of copper had little effect upon the distribution of ^{65}Zn among the nonintestinal tissues. If copper was given intraperitoneally either 2 or 18 hours before zinc administration, however, a significantly greater proportion of the absorbed ^{65}Zn was deposited in the liver and significantly smaller proportions were found in the blood, heart and kidneys.

A second index of ^{65}Zn absorption is the amount of the administered dose that disappeared from the ligated segment during the 3-hour experimental period. These values for percentage disappearance are presented in table 2. They are consistent with tissue uptake values presented in table 1, that is, the intraduodenal administration of copper depressed ^{65}Zn disappearance compared with the controls,

[3] All counting was done with a Nuclear-Chicago Model 4218 automatic gamma counter, Nuclear-Chicago Corporation, Des Plaines, Ill.

[4] Perkin-Elmer Model 303, Perkin-Elmer Company, Norwalk, Conn.

TABLE 1

Effect of method of copper administration on uptake and distribution of ^{65}Zn

Method of copper administration [1]	Dose in sampled tissues [2,3]	Relative distribution [4]			
		Blood [5]	Heart	Kidneys	Liver
	%				
Control (no Cu)	19.0 [a,6]	15.3 [a]	0.85 [a]	13.1 [a]	70.8 [a]
ID–0	7.5 [b]	16.0 [a]	1.10 [b]	12.5 [a]	70.3 [a]
IP–0	16.8 [a]	13.6 [a]	0.91 [ab]	12.9 [a]	72.7 [a]
IP–2	23.2 [a]	7.2 [b]	0.62 [c]	10.0 [b]	82.0 [b]
IP–18	33.6 [c]	7.8 [b]	0.61 [c]	8.0 [c]	83.8 [b]

[1] ID = intraduodenal; IP = intraperitoneal; 0, 2 and 18 indicate that copper was administered either simultaneously with ^{65}Zn, 2 hours prior to ^{65}Zn or 18 hours prior to ^{65}Zn, respectively.

[2] Percentage of dose taken up by blood, heart, kidneys and liver.

[3] Each value is the mean of eight observations.

[4] Relative distribution was calculated by dividing the counts per minute in a particular tissue by the counts per minute in all four sampled tissues.

[5] The total amount of ^{65}Zn in blood was calculated by multiplying counts per minute per milliliter by 0.07 × body weight.

[6] Values in any column that are not followed by the same letter are significantly different.

TABLE 2

Effect of method of copper administration on the absorption of ^{65}Zn from ligated segments and on the distribution of the remaining ^{65}Zn between the segment and its contents

Method of copper administration [1]	Disappearance of ^{65}Zn from ligated segment [2]	Distribution of remaining ^{65}Zn between segment and contents	
		Segment	Contents
	%		
Control (no Cu)	40.6 [a,3]	44.3 [a]	55.7 [a]
ID–0	28.3 [b]	65.3 [b]	34.8 [b]
IP–0	35.8 [ab]	50.0 [ab]	47.5 [ab]
IP–2	40.9 [a]	46.2 [a]	53.8 [a]
IP–18	55.3 [c]	56.6 [ab]	43.2 [ab]

[1] ID = intraduodenal; IP = intraperitoneal; 0, 2 and 18 indicate that copper was administered either simultaneously with ^{65}Zn, 2 hours prior to ^{65}Zn or 18 hours prior to ^{65}Zn, respectively.
[2] Each value is the mean of eight observations.
[3] Values in any column that are not followed by the same letter are significantly different.

whereas intraperitoneal administration of copper increased ^{65}Zn uptake if copper was given 18 hours prior to ^{65}Zn administration.

Because ^{65}Zn absorption was affected by copper treatment, the distribution of ^{65}Zn between the intestinal segment and its contents was of interest. The only significant effect on ^{65}Zn distribution between segment and contents was produced by intraduodenal copper administration. This treatment resulted in a significantly greater proportion of the total ^{65}Zn in the isolated loop being located in the intestinal tissue and a smaller proportion in the contents. This suggests that intraduodenally administered copper may have interfered with the normal movement of ^{65}Zn out of the intestinal epithelial cells.

Total copper levels of the sampled tissues are presented in table 3. Administration of copper intraduodenally resulted in statistically significant increases in the copper levels of the kidney, the intestinal segment and its contents. The intraperitoneal administration of copper significantly increased serum copper levels if administered simultaneously with ^{65}Zn, and increased kidney and liver copper levels in all cases.

The effects of copper on total zinc content of the tissues are presented in table 4. Intraduodenal administration of copper did not have any significant effect on zinc levels in the tissues sampled in these experiments. Intraperitoneal administration of copper tended to lower liver and kidney zinc levels as compared with the controls, although not all the differences were statistically significant. Copper administration intraperitoneally did cause a significant lowering of total serum zinc when given 18 hours prior to ^{65}Zn administration. The administration of copper did not have any significant effect on zinc content of the intestinal segments but tended to decrease the total amount of zinc in the contents.

DISCUSSION

In these experiments, the administration of copper to rats produced two major effects on ^{65}Zn absorption. When copper was administered intraduodenally along with ^{65}Zn, it depressed subsequent absorption of ^{65}Zn. When the same amount of copper was given intraperitoneally, 18 hours before ^{65}Zn was administered, subsequent absorption of ^{65}Zn was significantly greater than that of the controls. These two effects will be discussed in turn.

The depression of ^{65}Zn absorption by intraduodenally administered copper could be mediated in at least two different ways. The most obvious possibility is that copper impeded the absorption of zinc via a direct effect at the intestinal level, that is, it impeded either the entry of ^{65}Zn into the intestinal epithelial cells or the movement of ^{65}Zn from the intestinal mucosal cells into the general circulation. An alternate possibility is that copper is absorbed from the intestine and is deposited in the blood, kidneys, liver or some other nonintestinal tissue where it prevents subsequent uptake of ^{65}Zn. This could result in a "feedback" effect and thus impede movement of ^{65}Zn from the intestine into the general circulation.

The evidence provided by these short-term experiments favors the direct inhibition of ^{65}Zn absorption. When copper was

TABLE 3

Effect of method of copper administration on tissue copper levels

Method of copper administration [1]	Copper concentrations [2]						
	Serum	Erythrocytes	Heart	Kidneys	Liver	Segment	Contents
	$\mu g/ml$ [3]		$\mu g/g$ [4]			μg [5]	
Control (no Cu)	1.09 [a,6]	0.85 [a]	25.4 [a]	24.7 [a]	18.9 [a]	2.0 [a]	1.1 [a]
ID–0	1.21 [ab]	1.01 [a]	25.0 [a]	30.2 [b]	27.5 [a]	82.4 [b]	69.5 [b]
IP–0	1.51 [b]	1.09 [a]	24.7 [a]	31.0 [b]	49.3 [b]	13.5 [a]	3.7 [a]
IP–2	1.38 [ab]	1.16 [a]	23.8 [a]	30.3 [b]	50.6 [b]	3.3 [a]	3.0 [a]
IP–18	1.35 [ab]	1.00 [a]	24.2 [a]	34.2 [b]	61.0 [b]	4.4 [a]	5.1 [a]

[1] ID = intraduodenal; IP = intraperitoneal; 0, 2 and 18 indicate that copper was administered either simultaneously with ^{65}Zn, 2 hours prior to ^{65}Zn or 18 hours prior to ^{65}Zn, respectively.
[2] Each value is the mean of eight observations.
[3] Micrograms of copper per milliliter of packed red cells.
[4] Values for heart, kidneys and liver are on a dry weight basis.
[5] Values indicate total copper in the segment and its contents.
[6] Values in any column that are not followed by the same letter are significantly different.

TABLE 4

Effect of method of copper administration on tissue zinc levels

Method of copper administration [1]	Zinc concentrations [2]						
	Serum	Erythrocytes	Heart	Kidneys	Liver	Segment	Contents
	$\mu g/ml$ [3]		$\mu g/g$ [4]			μg [5]	
Control (no Cu)	1.84 [a,6]	1.51 [a]	83.0 [ab]	116.8 [a]	142.1 [a]	27.8 [a]	5.4 [a]
ID–0	1.83 [a]	1.45 [a]	84.7 [ab]	111.6 [ab]	136.2 [a]	29.6 [a]	4.2 [ab]
IP–0	1.96 [a]	1.42 [a]	80.6 [b]	110.3 [ab]	102.1 [b]	28.4 [a]	3.7 [b]
IP–2	1.71 [a]	1.58 [a]	81.8 [b]	100.5 [b]	121.2 [ab]	27.4 [a]	4.3 [ab]
IP–18	1.31 [b]	1.43 [a]	86.2 [a]	110.5 [ab]	100.5 [b]	29.0 [a]	4.0 [ab]

[1] ID = intraduodenal; IP = intraperitoneal; 0, 2 and 18 indicate that copper was administered either simultaneously with ^{65}Zn, 2 hours prior to ^{65}Zn or 18 hours prior to ^{65}Zn, respectively.
[2] Each value is the mean of eight observations.
[3] Micrograms of zinc per milliliter of packed red cells.
[4] Values for heart, liver and kidney are on a dry weight basis.
[5] Values indicate the total zinc in the segment and its contents.
[6] Values in any column that are not followed by the same letter are significantly different.

administered intraperitoneally, the levels of copper in such nonintestinal tissues as blood, heart, kidneys and liver were as high as, or higher than the levels attained by intraduodenal administration of copper, yet there was no inhibition of ^{65}Zn absorption in intraperitoneally treated rats. Further, it was found that intraduodenal administration of copper caused ^{65}Zn to accumulate in the intestinal segment but did not result in any abnormal accumulation of ^{65}Zn in any of the other tissues. All of these results would indicate that the depression in ^{65}Zn absorption produced by the intraduodenal administration of copper was not mediated by either a buildup of copper or by a depletion of zinc in nonintestinal tissues. Rather, the experiments reported here suggest that the depression in ^{65}Zn absorption that occurred following the intraduodenal administration of copper was mediated directly at the intestinal level. This is consistent with an earlier report (7) that zinc interfered with ^{64}Cu uptake from ligated segments, and this interference apparently was mediated either in or on the intestine. Thus, the combined results of the current report and the previous one (7) indicate that there is a mutual antagonism between zinc and copper during the absorption process, and this antagonism apparently takes place either in or on the intestinal epithelial cells.

The increase in ^{65}Zn absorption that was observed when copper was administered intraperitoneally, 18 hours before ^{65}Zn dosing, is difficult to explain; however, one possibility that is consistent with the observed data follows. The rats that received copper intraperitoneally 18 hours prior to ^{65}Zn administration had concentrations of zinc in the liver and serum that were significantly lower than those of the controls. These rats also had higher liver copper levels than the controls and the livers of these rats contained a significantly higher proportion of the absorbed ^{65}Zn than did the controls. These observations are all

consistent with the idea that intraperitoneal administration of copper caused mobilization of zinc in the liver, and possibly in other tissues as well, thus creating a zinc-depleted state in these tissues. It is known that animals whose tissues have been depleted of iron will absorb dietary iron more efficiently than iron-adequate animals (10). If this phenomenon also holds for zinc, then a copper-induced depletion of tissue zinc levels might also be expected to increase zinc absorption. The possibility that copper induces an increased capacity in some zinc transport system also is an intriguing possibility; however, there is no evidence at present for a specific transport system for zinc. Obviously, there are a number of alternatives for explaining the increased ^{65}Zn uptake by rats given copper intraperitoneally, but the actual reasons for the increase cannot be elucidated from the data obtained in these experiments.

LITERATURE CITED

1. Sutton, W. R., and V. E. Nelson 1937 Studies of zinc. Proc. Soc. Exp. Biol. Med., 36: 211.
2. Smith, S. E., and E. J. Larson 1946 Zinc toxicity in rats. Antagonistic effects of copper and liver. J. Biol. Chem., *163:* 29.
3. Gray, L. F., and G. H. Ellis 1950 Some interrelationships of copper, molybdenum, zinc and lead in the nutrition of the rat. J. Nutr., *40:* 441.
4. Grant-Frost, D. R., and E. J. Underwood 1958 Zinc toxicity in the rat and its interrelation with copper. Australian J. Exp. Biol. Med. Sci., *36:* 339.
5. Magee, A. C., and G. Matrone 1960 Studies on growth, copper metabolism and iron metabolism of rats fed high levels of zinc. J. Nutr., *72:* 233.
6. Van Campen, D. R. 1966 Effects of zinc, cadmium, silver and mercury on the absorption and distribution of copper-64 in rats. J. Nutr., *88:* 125.
7. Van Campen, D. R., and P. U. Scaife 1967 Zinc interference with copper absorption in rats. J. Nutr., *91:* 473.
8. Van Campen, D. R., and E. A. Mitchell 1965 Absorption of Cu^{64}, Zn^{65}, Mo^{99} and Fe^{59} from ligated segments of the rat gastrointestinal tract. J. Nutr., *86:* 120.
9. Duncan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics, *11:* 1.
10. Moore, C. V., and R. Dubach 1956 Metabolism and requirements of iron in the human. J. Amer. Med. Assoc., *162:* 197.

Factors Affecting Vitamin B_6 Requirement in the Rat as Determined by Erythrocyte Transaminase Activity [1,2]

OFELIA V. DIRIGE [3] AND JOHN R. BEATON
Department of Food and Nutritional Sciences, University of Hawaii, Honolulu, Hawaii

ABSTRACT The sensitivity of erythrocyte glutamic-pyruvic transaminase (GPT) activity for assessment of vitamin B_6 nutriture was demonstrated by a significant correlation between vitamin intake and GPT activity. The effects of dietary calorie, protein and amino acid intakes on vitamin B_6 requirement were investigated in male rats using erythrocyte GPT activity as a criterion of vitamin B_6 nutriture. There was no significant difference in erythrocyte GPT activity between hypothalamic-hyperphagic and control rats indicating that an excessive calorie intake does not alter apparent vitamin B_6 requirement. With a 40-μg pyridoxine intake daily, there was a decrease in erythrocyte GPT activity as the level of protein in the diet increased, indicating that vitamin B_6 requirement is apparently related to the level of dietary protein. Neither amino acid imbalance (8% casein + 15% gelatin) nor methionine toxicity (8% casein + 3% DL-methionine) appeared to alter vitamin B_6 requirement as judged by erythrocyte GPT activity.

Although dietary need for vitamin B_6 has been recognized (1, 2) in animal species including man, precise requirements have not been established nor have the factors that influence the requirement been determined. A major problem has been the lack of a precise and sensitive method to assess vitamin B_6 nutriture. Among the methods employed to measure vitamin B_6 status, the tryptophan load test has been used most widely although Williams (3) has outlined its several disadvantages. Cheney et al. (4) showed that the measurement of blood glutamic-pyruvic transaminase (GPT) activity as an index of vitamin B_6 nutriture in rats was more advantageous and at least as sensitive as the tryptophan load test. The use of erythrocyte GPT activity was found (5) to be of greater value than whole blood or plasma GPT activity since it was not affected by factors that alter plasma enzyme activities.

Major nutrients of the diet may influence vitamin requirements. Requirements for thiamine, riboflavin and niacin have been related to the calorie intake and the possibility of such a relationship for vitamin B_6 must be considered. It has been suggested (6–8) that vitamin B_6 requirement is dependent in part upon the protein content of the diet. Abnormal tryptophan metabolism as evidenced by increased xanthurenic acid excretion (6, 8) occurred more rapidly in men given a high protein diet than in men having a low protein intake. It was postulated that high protein diets are detrimental due to ingestion of large amounts of tryptophan. Cerecedo et al. (9) concluded that the deleterious effect of high protein diets was due to ingestion of large amounts of sulfur-containing amino acids, especially methionine.

The effects of calorie, protein and amino acid intakes on apparent vitamin B_6 requirement of the rat have been investigated in the present study using erythrocyte GPT activity as the criterion of vitamin B_6 nutriture. The validity and sensitivity of erythrocyte GPT activity as a measure of vitamin B_6 nutriture were investigated also.

MATERIALS AND METHODS

Adult male rats of the Fisher strain, with an initial body weight range of 149 to 199 g, were housed in individual screen-bottomed cages at an environmental tem-

Received for publication July 25, 1968.
[1] Supported in part by a grant from the Office of Research Administration, University of Hawaii.
[2] Published with the approval of the Director, Hawaii Agricultural Experiment Station as Technical Paper no. 990.
[3] Based upon a thesis submitted by Ofelia V. Dirige in partial fulfillment of the requirements for the M.S. degree at the University of Hawaii.

perature of 24 ± 1°. Drinking water was available ad libitum at all times. The compositions of the several isocaloric diets used in this investigation are shown in table 1.

In all experiments, body weights were measured twice each week and food intake was measured daily. At the end of the experimental period, the animals were anesthetized with pentobarbital sodium given by intraperitoneal injection in water (6 mg/100 g body weight) and blood was obtained by cardiac puncture using heparinized syringes. Erythrocytes were separated from the plasma by centrifuging for 15 minutes at 2500 rpm after which they were washed once in an equal volume of 0.9% saline, recentrifuged and the saline supernatant discarded. The volume of erythrocytes was measured and two volumes of distilled water were added and mixed. This hemolysate was stored in the frozen state. Samples were thawed, centrifuged and the supernatant analyzed for GPT activity. Measurement of erythrocyte GPT activity was carried out by a modification (10) of the method devised by Tonhazy et al. (11).

To produce hyperphagia in one experiment, animals were anesthetized by intraperitoneal injection of pentobarbital sodium (3 mg/100 g body weight), and bilateral radio frequency (30 rf ma for 30 seconds) lesions of the ventromedial area of the hypothalamus were made with a stereotaxic instrument.[4] The coordinates used for placing the electrodes were anterior 6 mm, vertical 8.6 mm and lateral 1 mm.

Stanadard errors (SE) were calculated for all means and Student's *t* test was applied to determine significance of differences between means. Correlation coefficients were determined in some experiments.

Relationship between erythrocyte GPT activity and vitamin B_6 intake. Forty-two rats were divided into six groups of seven animals each and were fed ad libitum with the 20% casein, pyridoxine-free diet for 7 days. The animals were then injected subcutaneously daily for 17 days with pyridoxine·HCl in water to provide 20, 50, 80, 120, 200 or 400 μg/rat per day. Food

[4] David Kopf, Box 636, Tujunga, California.

TABLE 1

Composition of the experimental basal diets in percent by weight

Constituent	8% casein	10% casein	20% casein	30% casein	40% casein	50% casein	60% casein	Amino acid imbalance	Methionine toxicity
Casein, vitamin-free	4	6	16	26	36	46	56	4	4
Vitamin powder [1]	4	4	4	4	4	4	4	4	4
Sucrose	37.7	36.7	31.7	26.7	21.7	16.7	11.7	30.2	36.2
Corn oil	10	10	10	10	10	10	10	10	10
Salts [2]	4	4	4	4	4	4	4	4	4
Choline	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Inositol	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Alphacel	2	2	2	2	2	2	2	2	2
Cornstarch	37.7	36.7	31.7	26.7	21.7	16.7	11.7	30.2	36.2
Gelatin	—	—	—	—	—	—	—	15	—
DL-Methionine	—	—	—	—	—	—	—	—	3

[1] Contained: (in milligrams/800 g casein) thiamine·HCl, 100; niacin, 900; riboflavin, 200; *p*-aminobenzoic acid, 400; Ca pantothenate, 400; biotin, 20; folic acid, 20; menadione, 10; α-tocopherol, 2000; vitamin A palmitate, 400; and vitamin D_2, 50.

[2] P. H. Phillips, and E. B. Hart 1935 J. Biol. Chem., *109:* 657.

intake was limited to 13 g of the 20% casein, pyridoxine-free diet per rat per day for all animals to eliminate differences in food intake. All animals were killed on day 17 and erythrocytes were analyzed for GPT activity.

Vitamin B_6 and calorie intake. Thirty rats were divided into three groups of 10 animals each; the 10 rats of group A were hypothalamic-hyperphagic. All groups were fed the 20% casein diet ad libitum for 7 days after which they were provided with a basal 20% casein, pyridoxine-free diet for 21 days. The levels of food intake were controlled to eliminate intragroup variations in food intake — high intake (25 g/rat per day) for group A; normal (17 g/rat per day) for group B; and restricted (10 g/rat per day) for group C. Pyridoxine·HCl was added daily in water to the diet of all groups in the amount of 40 μg/rat per day. The rats were killed on day 21 and erythrocytes were analyzed for GPT activity.

Vitamin B_6 and protein intake. Eighty rats were divided into two groups of 40 animals each. Group 1 was given 40 μg pyridoxine·HCl/rat per day and group 2 was given 10 μg/rat per day, the vitamin being fed in the diet. Each group was further divided into five subgroups provided with different levels of protein (10, 30, 40, 50 or 60% by weight) and protein additions were made at the expense of sucrose. Food intake was limited to 13 g/day to eliminate group differences. After 14 days, the animals were killed and erythrocyte GPT activities were determined.

Vitamin B_6 and dietary amino acid imbalance and toxicity. Thirty-five rats were divided into five groups with seven animals in each group. All were fed a basal 8% casein diet for 2 days. For the next 14 days, three groups were provided with the 8% casein diet, one group with 8% casein + 15% gelatin and one group with 8% casein + 3% DL-methionine; gelatin and methionine were added at the expense of sucrose. Of the three groups fed the 8% casein diet, one was fed ad libitum, one was pair-fed with the gelatin group and one was pair-fed with the methionine group. Pyridoxine·HCl was added to all diets at a level of 15 μg/10 g diet. After 14 days the animals were killed and erythrocyte GPT activities were measured.

RESULTS

Relationship between erythrocyte GPT activity and vitamin B_6. Erythrocyte GPT activity (table 2) increased with an increasing daily intake of vitamin B_6, a significant positive correlation between dosage and erythrocyte GPT response being observed ($r = +0.787$; $P < 0.05$) in the daily dosage range 20 to 400 μg pyridoxine. Maximal erythrocyte GPT activity was observed at a daily dose of 200 μg with the level of activity being comparable at the higher dose of 400 μg.

Vitamin B_6 and calorie intake. The results of this experiment are set down in

TABLE 2

Relationship between erythrocyte GPT activity and vitamin B_6 intake [1]

Group [2]	Vitamin B_6 intake	Avg body wt gain	GPT activity
	μg/rat/day	*g*	*μg pyruvate/ml erythrocyte*
A	400	99.3 ± 2.89	92.5 ± 3.86
B	200	96.1 ± 2.78	101.1 ± 3.42
C	120	92.6 ± 2.21	73.1 ± 3.46
D	80	98.1 ± 1.82	73.3 ± 1.58
E	50	94.4 ± 0.82	65.8 ± 3.57
F	20	90.3 ± 3.18	55.7 ± 3.80
		C versus D; $P < 0.05$	A versus D; $P < 0.02$
		C versus A; $P < 0.02$	A versus E; $P < 0.01$
			A versus F; $P < 0.001$
			B versus E; $P < 0.001$
			D versus F; $P < 0.02$

[1] Results expressed as mean ± SE of the mean for seven male rats per group.
[2] All groups were provided with 13 g/rat per day of the 20% casein diet for a period of 17 days.

table 3. Erythrocyte GPT activity was significantly higher in food-restricted animals than in either control or hyperphagic animals. There was no significant difference in erythrocyte GPT activity between hyperphagic and control animals. Thus, it would appear that an excessive calorie intake does not cause an increase in apparent vitamin B_6 requirement.

Vitamin B_6 and protein intake. Results are shown in table 4 and figure 1. With a 10-μg pyridoxine intake, there was an increasing body weight gain up to the 40% protein dietary level, after which a plateau was attained. Above the 40% protein level, vitamin B_6 intake appeared to be limiting so that no further gain in weight was observed. With a 40-μg pyridoxine intake, body weight gain increased as the level of protein in the diet increased to 60%; in this situation, the vitamin intake was not limiting. There was no significant difference in average body weight gains between groups fed 40 or 10 μg pyridoxine at any level of dietary protein in the 10 to 60% range. It should be noted, however, that

TABLE 3

Vitamin B_6 and calorie intake [1]

Group [2]	Pyridoxine·HCl	Daily food intake	Avg body wt gain	GPT activity
	μg/g food	*g/rat*	*g*	*μg pyruvate/ml erythrocyte*
A (hyperphagic)	1.6	25	166 ± 6.61	39.2 ± 0.98
B (normal)	2.35	17	143 ± 0.98	33.0 ± 1.02
C (restricted)	4.0	10	63 ± 1.02	59.6 ± 3.33
			A versus C; $P < 0.001$	A versus C; $P < 0.02$
			B versus C; $P < 0.001$	B versus C; $P < 0.01$

[1] Results expressed as mean ± SE of the mean for 10 male rats/group.
[2] All groups were provided with the 20% casein diet ad libitum for 21 days. Pyridoxine·HCl was added to the food at a level to provide 40 μg/rat per day.

TABLE 4

Vitamin B_6 and protein intake [1]

Group [2]	Treatment	Vitamin B_6 intake	Avg body wt gain	GPT activity
		μg/day	*g*	*μg pyruvate/ml erythrocyte*
A (8) [3]	10% casein	10	32 ± 4.26	41.4 ± 3.92
C (8)	30% casein	10	48 ± 4.23	35.0 ± 4.21
E (8)	40% casein	10	55 ± 3.33	29.8 ± 0.87
G (8)	50% casein	10	55 ± 5.90	33.9 ± 3.12
I (8)	60% casein	10	50 ± 4.25	36.3 ± 3.46
B (8)	10% casein	40	28 ± 3.50	73.4 ± 5.22
D (8)	30% casein	40	42 ± 5.10	67.2 ± 4.46
F (8)	40% casein	40	44 ± 4.15	66.3 ± 4.15
H (8)	50% casein	40	53 ± 3.98	48.8 ± 1.84
J (8)	60% casein	40	65 ± 3.59	52.4 ± 2.27
		A versus E, G; $P < 0.01$		B versus H; $P < 0.05$
		B versus H; $P < 0.01$		D versus H; $P < 0.05$
		B versus J; $P < 0.001$		F versus H; $P < 0.05$
				A versus B; $P < 0.02$
				C versus D; $P < 0.01$
				E versus F; $P < 0.001$
				G versus H; $P < 0.01$
				I versus J; $P < 0.01$

[1] Results expressed as mean ± SE of the mean.
[2] All groups were provided with 13 g food/rat per day for a period of 14 days.
[3] Numbers in parentheses indicate numbers of animals in group.

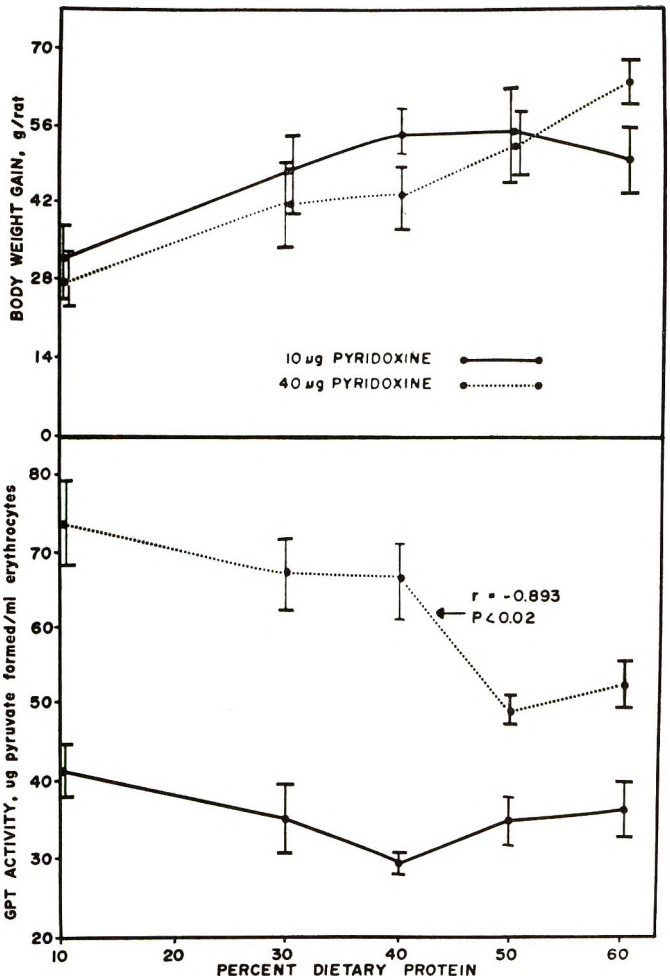


Fig. 1 Average body weight gain and erythrocyte GPT activity of rats fed 10 and 40 μg pyridoxine/rat per day with varying levels of protein intake for 14 days. Each point is the mean value and the SE of the mean is shown by vertical bars.

food intake was restricted to 13 g/rat per day in all groups and this could prevent marked differences in body weight gain. With a pyridoxine intake of 10 μg, there was no significant effect on erythrocyte GPT activity of dietary protein level. With a 40-μg pyridoxine intake, there was a significant negative correlation ($r = -0.893$; $P < 0.02$) between erythrocyte GPT activity and the dietary level of protein. At all levels of dietary protein intake, animals fed 40 μg pyridoxine had greater erythrocyte GPT activities than did those fed 10 μg pyridoxine.

Vitamin B_6 and dietary amino acid imbalance and toxicity. As shown in table 5, group A (8% casein ad libitum) showed the greatest gain in body weight. The group fed the 8% casein + 15% gelatin gained weight, whereas its pair-fed control lost weight, indicating perhaps that some part of the gelatin may have been used for tissue synthesis. The limiting amino acid in casein is methionine and it is possible that the addition of gelatin, which contains 0.051 g methionine/g total nitrogen, may have supplemented the casein sufficiently to allow a small increase in body weight.

TABLE 5

Vitamin B_6 and dietary amino acid imbalance and toxicity [1]

Group	Treatment	Avg daily food intake	Avg vitamin B_6 intake	Avg body wt change	GPT activity
		g	*μg/day*	*g*	*μg pyruvate/ml erythrocyte*
A	8% casein, ad libitum	14.3 ± 0.47	20	+26 ± 2.76	75.2 ± 4.24
B	8% casein, pair-fed with D	9	13.5	− 7 ± 1.30	58.5 ± 5.04
C	8% casein, pair-fed with E	7	10.5	−19 ± 2.28	55.7 ± 4.89
D	8% casein + 15% gelatin	9	13.5	+ 4 ± 2.78	67.8 ± 6.22
E	8% casein + 3% DL-methionine	7	10.5	−16 ± 2.52	62.0 ± 4.46
				A versus B; $P < 0.01$	
				A versus C; $P < 0.001$	
				B versus D; $P < 0.02$	

[1] Results expressed as mean ± SE of the mean for seven male rats per group fed for a period of 14 days.

The data in table 5 show that the spontaneously lower vitamin B_6 intake of groups B and C was associated with reduced erythrocyte GPT activity. The amino acid imbalance (casein plus gelatin to a total of 23% protein) had no effect on the erythrocyte GPT activity and hence, on apparent vitamin B_6 requirement. The data show also that methionine toxicity (addition of 3% DL-methionine) did not alter vitamin B_6 requirement as assessed by erythrocyte GPT activity.

DISCUSSION

The sensitivity and validity of erythrocyte GPT activity as a criterion of vitamin B_6 nutriture were tested in these experiments. Erythrocyte GPT activity increased with an increasing dose of pyridoxine within the range of 20 to 200 μg with a maximal activity at a daily dose of about 200 μg and a comparable activity at the higher dose of 400 μg (table 2). The results of Beaton and Cheney (12) have indicated that in rats, the requirement for maximal erythrocyte GPT activity is at least 80 μg/day. An intake of 40 μg pyridoxine/rat per day was associated with a higher erythrocyte GPT activity at all levels of protein intake than an intake of 10 μg pyridoxine (table 4). The animals fed an 8% casein diet ad libitum, with a pyridoxine intake of 20 μg pyridoxine/rat per day had a slightly (but not significant) higher erythrocyte GPT activity than the group receiving an intake of 10.5 pyridoxine/rat per day (table 5). These observations indicate that erythrocyte GPT activity reflects the level of vitamin B_6 intake in the diet; hence, this method appears to be a sensitive test for assessment of vitamin B_6 nutriture. It might be reasoned that GPT, once formed, should remain fairly constant in the erythrocyte although obviously affected by erythrocyte turnover rate. One might then consider that the apoenzyme would be relatively less affected by dietary variables and GPT activity would then reflect vitamin B_6 nutriture in a sensitive manner. The results of these experiments indicate that this may be so although a firm understanding of the mechanism involved cannot be reached from the present data. Further, Heddle et al. (13) observed that a 40-fold increase in pyridoxine·HCl administration in the rat did not cause an in vivo "induction" of enzyme activity; this supports the conclusion that erythrocyte transaminase activity is a sensitive indicator of vitamin B_6 nutriture.

Hyperphagic and control rats with the same dietary intake of pyridoxine had no significant difference in mean erythrocyte GPT activity despite a 47% difference in calorie intake (table 3). This suggests that an excessive food (and calorie) intake does not seem to alter erythrocyte GPT activity or apparent vitamin B_6 requirement. The increased erythrocyte GPT activity of food-restricted rats may indicate a decreased vitamin B_6 requirement with a decreased calorie intake. It may indicate, however, the effect of a relative catabolic state and decreased protein synthesis; these have been shown to increase tissue transaminase activity (14). Cheney et al. (4) have reported increased liver and blood GPT activities with increasing severity of

food restriction. An alternative explanation is offered by the calculated data of vitamin B_6 intake per gram food intake (table 3). The food-restricted group had a markedly higher dietary vitamin concentration which could conceivably account for the higher erythrocyte GPT activity. It should be noted that in the present experiment, animals received an adequate but not excessive intake (40 μg/day) of pyridoxine; if the vitamin intake had been minimal, different results might have been obtained.

Our observations with varying dietary protein levels (table 4) fed with some degree of restriction to eliminate intragroup differences, indicate that a daily intake of 10 μg pyridoxine is inadequate to promote increasing weight gain at high levels of protein intake and, hence, the vitamin becomes limiting as dietary protein level increases. Because the groups given 40 μg pyridoxine/day increased in body weight as dietary protein increased from 10 to 60%, it appears that an intake of 40 μg pyridoxine/day is adequate to promote body weight gain even under conditions of a high level of protein intake with some degree of food restriction. Beaton and Cheney using rats fed ad libitum (12) reported that pyridoxine·HCl requirement for maximal weight gain was at least 40 μg/day and may approximate 80 μg/day. Vitamin B_6 concentration of liver was found (15) to be fairly constant at an intake between 15 to 25 μg pyridoxine·HCl /rat per day while the average body weight gain was still increasing at the 25-μg dose level, suggesting that the amount required for maximal body weight is greater than 25 μg and greater than that required for tissue saturation. Brin and Thiele (16) concluded that a dietary level of 2.0 μg/g diet of pyridoxal was adequate to support maximal growth.

With a pyridoxine intake of 10 μg, there was no significant alteration in erythrocyte GPT activity as the protein level of the diet increased from 10 to 60% (table 4), perhaps due to the low level of pyridoxine intake preventing a further lowering of enzyme activity with increasing dietary protein levels. With a 40-μg pyridoxine intake, the significant negative correlation between erythrocyte GPT activity and level of protein intake suggests that vitamin B_6 requirement is inversely related to the level of dietary protein. Other studies (6, 17, 18) support this conclusion. Cheney et al. (4), however, failed to observe any effect of dietary protein level in the 5 to 40% range on whole blood GPT activity.

Our observations (table 5) suggest that a gross amino acid imbalance does not affect apparent vitamin B_6 requirement as assessed by erythrocyte GPT activity. Further, it should be noted that the gelatin-supplemented diet provided a total protein intake of 23% by weight of which 15% was in the form of an incomplete protein. Under these conditions, an increase of dietary protein intake (from 8 to 23%) did not appear to alter vitamin B_6 nutriture; this is in contrast to the increased requirement apparent when the dietary level of a complete protein (casein) was increased. It would appear that the effect of dietary protein on vitamin B_6 requirement is dependent upon both the quantity and quality of the dietary protein. It is of interest that substitution of gelatin for casein in a pyridoxine-free diet has been reported to prevent the appearance of acrodynia, the external manifestation of vitamin B_6 deficiency in rats (19). No significant difference was noted between the erythrocyte GPT activities of the groups pair-fed the 8% casein and the methionine-supplemented diet although 3% level of methionine in a low protein diet is reported to be toxic to the rat (20). This suggests that methionine toxicity does not increase vitamin B_6 requirement, in contrast with previous conclusions (7, 21) which were, however, based upon experiments in which methionine was added to diets of higher protein content.

Considering the results obtained in these investigations, it is concluded that the determination of erythrocyte GPT activity is a sensitive measure of vitamin B_6 nutriture since the level of enzyme activity reflects the dietary intake of the vitamin. Using this method as a criterion to assess vitamin B_6 status, it would appear that an excessive calorie intake does not alter erythrocyte GPT activity and thus, would not appear to affect vitamin B_6 requirement. Further, our findings suggest that dietary amino acid imbalance and methionine toxicity do not alter vitamin B_6 require-

ment. Results of this investigation support the hypothesis that vitamin B_6 requirement is directly related to the level of dietary protein provided that the protein is complete; addition of an incomplete protein (gelatin) does not alter the apparent vitamin requirement.

ACKNOWLEDGMENT

The authors express their appreciation to Miss Mary Jane Gooley for her valuable technical assistance throughout the course of these experiments, and to the Center for Cultural and Technical Interchange between East and West for personal support for Ofelia V. Dirige.

LITERATURE CITED

1. Coursin, D. 1961 Present status of vitamin B_6 metabolism. Amer. J. Clin. Nutr., 9: 304.
2. Sebrell, W. H. 1964 The importance of vitamin B_6 in human nutrition. Vitamins Hormones, 22: 875.
3. Williams, M. 1967 Present knowledge of vitamin B_6. In: Present Knowledge of Nutrition, ed. 3. Nutrition Foundation, New York, p. 66.
4. Cheney, M., D. Curry and G. H. Beaton 1965 Blood transaminase activities in vitamin B_6 deficiency: specificity and sensitivity. Can. J. Physiol. Pharmacol., 43: 579.
5. Cheney, M., Z. I. Sabry and G. H. Beaton 1967 Blood transaminase activities in vitamin B_6 deficiency: effect of depletion and repletion on the erythrocyte enzymes. Can. J. Physiol. Pharmacol., 45: 343.
6. Miller, L., and H. Linkswiler 1967 Effect of protein intake on the development of abnormal tryptophan metabolism by men during vitamin B_6 depletion. J. Nutr., 93: 53.
7. Cerecedo, L., and E. DeRenzo 1950 Protein intake and vitamin B_6 deficiency in the rat. II. The effect of supplementing a low protein, vitamin B_6 deficient diet with tryptophan and with other sulfur-free amino acids. Arch. Biochem., 29: 273.
8. Baker, E., J. E. Canham, W. T. Nunes, H. Sauberlich and M. E. McDowell 1964 Vitamin B_6 requirement for adult man. Amer. J. Clin. Nutr., 15: 59.
9. Cerecedo, L., J. Foy and E. DeRenzo 1948 Protein intake and vitamin B_6 deficiency in the rat. II. The effect of supplementing a low-protein, pyridoxine deficient diet with cystine or with methionine. Arch. Biochem., 17: 397.
10. Wroblewski, F., and P. Cabaud 1957 Colorimetric measurement of serum glutamic-pyruvic transaminase. Amer. J. Clin. Pathol., 27: 235.
11. Tonhazy, N., N. White and W. Umbreit 1950 A rapid method for the estimation of the glutamic-aspartic transaminase in tissues and its application to radiation sickness. Arch. Biochem., 28: 36.
12. Beaton, G. H., and M. Cheney 1965 Vitamin B_6 requirement of male albino rat. J. Nutr., 87: 125.
13. Heddle, J. G., E. W. McHenry and G. H. Beaton 1963 Penicillamine and vitamin B_6 interrelationships in the rat. Can. J. Biochem. Physiol., 41: 1215.
14. Beaton, J. R. 1963 Nitrogen metabolism in cold-exposed rats. Can. J. Biochem. Physiol., 41: 1169.
15. Beaton, G. H., and E. W. McHenry 1953 Vitamin B_6 levels in rat tissues. Brit. J. Nutr., 7: 357.
16. Brin, M., and V. F. Thiele 1967 Relationships between vitamin B_6-vitamer content and the activities of two transaminase enzymes in rat tissues at varying levels of vitamin B_6. J. Nutr., 93: 213.
17. Tulpule, P. G., and J. N. Williams 1955 The relationship of dietary protein and food intake to pyridoxine nutrition in the rat. J. Nutr., 57: 529.
18. Kelsay, J., A. Baysal and H. Linkswiler 1968 Effect of vitamin B_6 depletion on the pyridoxal, pyridoxamine and pyridoxine content of the blood and urine of men. J. Nutr., 94: 490.
19. Beaton, J. R., J. L. Beare and E. W. McHenry 1952 Factors affecting the development of acrodynia in pyridoxine-deficient rats. J. Nutr., 48: 325.
20. Beaton, J. R. 1967 Methionine toxicity in rats exposed to cold. Can. J. Physiol. Pharmacol., 45: 329.
21. DeBey, H. J., E. E. Snell and C. A. Baumann 1952 Studies on the interrelationship between methionine and vitamin B_6. J. Nutr., 46: 203.

Effect of D-Penicillamine Treatment on Mineral Balance in Guinea Pigs [1]

M. I. DJAFAR, H. R. CAMBEROS AND G. K. DAVIS
Division of Biological Sciences, University of Florida, Gainesville, Florida

ABSTRACT Experiments were conducted to study the effect of D-penicillamine on the metabolism of iron, magnesium, calcium and phosphorus in guinea pigs as measured by changes in absorption, retention and excretion of these elements. D-Penicillamine administration for 10 days did not change the iron and magnesium metabolisms as compared with the controls. Calcium absorption and urinary excretion were increased, but 50% of the guinea pigs had a negative calcium balance during the penicillamine treatment. A decreased phosphorus absorption and an increased urinary excretion were observed during the penicillamine treatment. Only 3 out of 16 guinea pigs, however, were in a negative phosphorus balance. The body weight of the guinea pigs was also decreased during the penicillamine administration, though feed consumption remained unchanged. The increased calcium and phosphorus urinary excretion, the loss of body weight and the normal blood levels of calcium and phosphorus suggested a decreased tubular reabsorption of these two minerals and the mobilization of calcium and phosphorus from the bone stores.

It is well known that penicillamine treatment causes a high increase in urinary copper excretion in humans (1, 2) and in guinea pigs (3). In humans, penicillamine is used as a treatment for Wilson's disease (4, 5) with good success in decreasing the copper accumulation in the body. Usually this penicillamine treatment for Wilson's disease requires months, even years to decrease the copper level in the organs to normal. Because of this prolonged treatment it is imperative to ascertain whether penicillamine affects the balance of other minerals besides copper. Not much is known about the effect of penicillamine on the balance of iron, magnesium, calcium and phosphorus. It was found that D-penicillamine formed relatively stable chelates with copper, mercury, nickel, zinc, lead, cobalt, cadmium and manganese (6). Penicillamine also can be used in the treatment of lead toxicity (7) and mercury poisoning (8).

Litin et al. (9) reported no significant changes in the urinary phosphorus of two patients with Wilson's disease and two normal subjects treated with penicillamine. There was a slight increase in urinary calcium of three patients with Wilson's disease after they were treated with penicillamine and one patient had a decrease in calcium excretion. In another study the same workers (10) did not find a significant change in urinary calcium excretion in normal subjects treated with penicillamine. In one case of hemosiderosis, penicillamine doubled the urinary iron excretion 3 days after its administration but in a case of hemochromatosis penicillamine treatment did not increase the urinary iron excretion (11). As far as we know, no other reports have been published, regarding the effect of penicillamine on iron or magnesium balance in normal subjects or animals. Because very few metabolic studies have been carried out with penicillamine, a series of balances was planned to study the effect of penicillamine on iron, magnesium, calcium and phosphorus in normal guinea pigs.

METHODS AND MATERIALS

Twenty-six young, male guinea pigs of the Hartley strain were purchased [2] and caged in stainless steel cages with stainless steel wire floors. Two groups of eight animals each were put into the metabolic cages which were also made from stainless steel. Two other groups of five animals each served as controls. Guinea pig laboratory

Received for publication June 27, 1968.
[1] Supported in part by Public Health Service Research Grant no. AM-09089 from the National Institutes of Health.
[2] Simonsen Lab. Incorporated, White Bear, Minnesota.

ration [3] was fed during the adjustment period and during the experiment. It contained 0.404 mg iron, 9.95 mg calcium and 2.75 mg magnesium/g of feed as analyzed by the atomic absorption spectrophotometer, and 8.7 mg phosphorus as analyzed according to Fiske and Subbarow (12).

D-Penicillamine [4] was administered orally (150 mg/day) for a period of 10 days. Guinea pig weights at the time of D-penicillamine administration were 382 to 432 g in experiment 1 and 524 to 737 g in experiment 2. Daily (24 hours) urine collection was performed before penicillamine treatment for a period of 4 days (control period) and during penicillamine treatment for 10 days (experimental period). The daily fecal collection was pooled from each animal for the control and the experimental period. Each pooled fecal sample was analyzed and the total amount was divided by the number of days to obtain the daily fecal excretion of the elements. The urine was diluted 1:1 with 10% trichloroacetic acid, filtered, and stored in the refrigerator (1 to 3°) for further determination. Feed, fecal samples and tissue samples were first dried overnight in an oven [5] at 110° until a constant weight was obtained, and later ashed in a furnace at 600° for 16 hours. The ashed samples were digested for 1 hour on a hot plate with 50% concentrated HCl, filtered, and diluted to a 50-ml volume with triple distilled water.

The animals were killed 24 hours after the last dose of D-penicillamine, and blood, liver, kidney and spleen were collected. Hemoglobin was determined by the cyanmethemoglobin method using a colorimeter/spectrophotometer.[6] Determinations of iron, magnesium and calcium in plasma, feed, urine, feces and organs were performed with an atomic absorption spectrophotometer [7] and phosphorus determinations were made with the colorimeter/spectrophotometer.[8] Lanthanum oxide (99.99%) [9] was used in the calcium and magnesium determinations to prevent interference by other elements, especially phosphorus. Lanthanum oxide was also added to the standards and blanks. Analysis of variance of the data was performed on the basis of a two-factorial and a single variable of classification (13).

RESULTS

Feed consumption although slightly reduced was not significantly affected by the oral administration of penicillamine, i.e., 24.5 g/day in the control period and 23.0 g/day in the experimental period (table 1). There were no significant changes in the apparent absorption, urinary excretion and retention of iron during the penicillamine treatment (tables 2 and 3). The iron levels in liver, spleen and kidney were not altered compared with the controls (table 4). As a result of the sampling technique, hemolysis occurred in most of the samples; therefore, plasma iron was not determined.

Magnesium balance was not affected by D-penicillamine treatment (tables 2 and 3) and no changes were observed in the concentration of magnesium in plasma (table 1) and organs (table 4).

Calcium balance was affected by D-penicillamine treatment (tables 2 and 3). Its

TABLE 1

Hemoglobin and hematocrit values in blood, mineral contents of plasma, feed consumption and body weight gain in 16 penicillamine-treated and 10 control animals.

	Control	Penicillamine-treated
Hemoglobin, g/100 ml	14.5 ± 1.0 [1]	15.4 ± 0.4
Hematocrit	46.4 ± 0.4	43.6 ± 1.0
Magnesium, mg/100 ml	4.3 ± 0.3	4.1 ± 0.2
Calcium, mg/100 ml	12.4 ± 0.3	11.8 ± 0.4
Phosphorus, mg/100 ml	8.1 ± 0.3	8.3 ± 0.2
Feed consumption, g/day	24.5 ± 0.9	23.0 ± 1.6
Body weight gain, g/day	4.1 ± 0.57	−2.01 ± 0.38

[1] SE of the mean.

[3] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis, Missouri.
[4] Nutritional Biochemicals Corporation, Cleveland, Ohio.
[5] Thelco double wall oven with thermostat, Curtin Company, Jacksonville, Florida.
[6] Spectronic 20, Bausch & Lomb, Scientific Instrument Division, Rochester, New York.
[7] Perkin-Elmer 303, Perkin-Elmer Corporation, Norwalk, Connecticut.
[8] See footnote 6.
[9] K & K Laboratories Incorporated, Plainview, New York.

TABLE 2

Effect of D*-penicillamine on daily intake, absorption, excretion and retention of iron, magnesium, calcium and phosphorus in guinea pigs*

Exp. no.	Iron				Magnesium				Calcium				Phosphorus			
	1		2		1		2		1		2		1		2	
No. of animals	8		8		8		8		8		8		8		8	
Period of time	C[1]	P[2]	C	P	C	P	C	P	C	P	C	P	C	P	C	P
Intake, mg	9.09	8.37	10.99	9.79	62.45	57.54	75.52	67.30	226.0	208.2	273.4	243.6	197.6	182.0	239.0	213.0
Fecal, mg	7.24	6.62	9.00	8.06	14.85	13.21	17.09	15.48	126.9	106.7	146.9	121.2	104.4	139.5	144.4	160.0
Absorbed, mg	1.85	1.75	1.99	1.73	47.60	44.33	58.43	51.82	99.1	101.5	126.5	122.4	93.2	42.5	94.6	53.0
Absorption, % of intake	20.29	20.88	18.10	17.77	76.22	77.04	77.37	77.00	43.85	48.75	46.26	50.26	47.15	23.39	39.62	24.98
Urinary, mg	0.16	0.12	0.20	0.15	30.09	32.09	38.69	33.19	53.96	98.51	80.70	117.70	27.59	33.69	29.48	34.99
Urinary, % of intake	1.78	1.43	1.77	1.53	48.18	55.77	51.23	49.31	23.87	47.32	29.52	48.33	13.96	18.51	12.33	16.43
Retention, mg	1.68	1.63	1.80	1.59	17.51	12.24	19.74	18.64	45.16	2.99	45.78	4.71	65.60	8.89	65.22	18.01
Retention, % of intake	18.51	19.45	16.34	16.24	28.04	21.27	26.14	27.69	19.98	1.44	16.74	1.93	33.19	4.88	27.29	8.46

[1] C identifies period before penicillamine administration.
[2] P identifies period during penicillamine administration.

apparent absorption (percentage of intake) was significantly increased ($P < 0.05$) and the urinary excretion (percentage of intake) was also significantly increased ($P < 0.01$). Although there was an increase in calcium absorption ($P < 0.05$), the urinary excretion was so much greater that 50% of the guinea pigs had a negative balance. The calcium level in the plasma of the treated animals was normal and did not differ significantly from the controls (table 1). The apparent phosphorus absorption (percentage of intake was significantly lower ($P < 0.01$) during the experimental period compared with the period before penicillamine administration. The urinary phosphorus excretion was significantly higher ($P < 0.01$) during the penicillamine treatment (tables 2 and 3). Although there were two negative factors in the phosphorus metabolism (less absorption and increased urinary excretion), only 3 animals out of 16 showed a negative phosphorus balance, compared with 50% evidencing a negative calcium balance. No changes were observed in the plasma phosphorus of the treated animals compared with the controls (table 1). The calcium and phosphorus concentrations in liver, spleen and kidney were similar in treated and control animals (table 4). There were no significant differences in the hemoglobin content and hematocrit values between the treated animals and the controls 24 hours after the last dose of penicillamine (table 1).

During the 10-day penicillamine treatment, the guinea pigs were losing weight, an average loss of 2.01 g/day, whereas feed consumption was only slightly decreased, an average of 1.5 g/day (table 1).

DISCUSSION

Penicillamine treatment caused taste abnormalities in humans that usually occurred within 3 to 6 weeks after initiation of the therapy. To prevent abnormal taste sensation, more salt or sweets were added to the diet to maintain the food intake. This abnormality persisted as long as the drug was given; however, upon discontinuation of the drug, subjective taste returned to normal within a few weeks. Most patients reported a loss of interest in eating (14). This abnormality was also observed by

TABLE 3

Analysis of variance of absorption (percentage of intake) and excretion (percentage of intake) of iron, magnesium, calcium and phosphorus

Source of variation	d.f.	Mean squares							
		Absorption				Excretion			
		Fe	Mg	Ca	P	Fe	Mg	Ca	P
Total	31								
Replication	1	110.26	3.32	28.69	139.87	0.0007	15.96	88.78	34.86
Treatment	1	264.50	0.58	151.81 *	2,732.46 **	0.4632	71.40	3,538.51 **	170.20 **
Interaction	1	23.12	1.75	1.32	248.08	0.0306	164.41	43.47	1.53
Within	1	91.56	26.59	26.55	59.85	0.1818	62.29	60.16	13.85

* $P < 0.05$.
** $P < 0.01$.

Sternlieb and Scheinberg (15), but they reported that within 6 months, despite continuation of the drug, it was resolved spontaneously.

In guinea pigs oral administration of D-penicillamine caused a drop in feed and water consumption on the first 2 days (6 out of 16 animals), but became normal on day 3 when the animals became accustomed to the penicillamine. Initially the amount of urine was slightly decreased during the penicillamine treatment; however, after 3 days it returned to a level similar to that of the pretreatment period.

Several workers found roentgenographic evidence of osteoporosis (16), of skeletal degeneration (17) and of osteomalacia (18) in patients with Wilson's disease. However, Randall et al. (19) did not find evidence of osteomalacia or osteoporosis in any of their nine patients. They and others (6, 9, 10, 20, 21) did observe an increase of urinary calcium in patients with Wilson's disease. They found no significant elevation of the serum calcium level. It is still not clear whether the hypercalciuria caused by Wilson's disease is the result of renal tubular defect, an increased absorption of calcium from the gastrointestinal tract, a skeletal disease or a combination of these possibilities (19). Wilson's disease is presently treated by chelating agents, especially penicillamine, with good success. The prolonged use of these drugs, however, may have an effect on the balance of other biologically active elements. It was shown (9, 10) that penicillamine treatment caused an increased calcium excretion in three patients, but a decrease in calcium excretion was observed in one patient. In normal subjects (two patients) the administration of penicillamine did not significantly alter the urinary calcium and phosphorus excretion. In this experiment with guinea pigs penicillamine definitely resulted in an increase in urinary calcium and phosphorus excretion. The number of animals having a negative balance of calcium and phosphorus was pronounced (respectively, 50 and 20%) in an experimental period of only 10 days. Over a longer period the negative balance of phosphorus could be increased, due to the simultaneous decrease of absorption and an increase of excretion, although there is a possibility these animals might adjust. Intravenous administration of ethylenediaminetetraacetate (EDTA) resulted in an increased urinary calcium excretion mostly as the complexed form, Ca-EDTA, and was accompanied by a phosphaturesis equivalent to the amount of calcium excreted in the complexed form (22).

The calcium and phosphorus levels in the plasma remained unchanged throughout the experimental period. The plasma calcium and phosphorus at no time fell below control values, suggesting that sufficient calcium and phosphorus were mobilized from stores, presumably skeletal, to prevent hypocalcemia or hypophosphatemia. Analyzing all observations, that is, urinary calcium and phosphate losses, normal blood levels of the two minerals and the subsequent decrease of the body weight, it appears likely that the increased

TABLE 4

Iron, magnesium, calcium and phosphorus in organs of 16 D-penicillamine-treated guinea pigs

Minerals	Treatments	Liver		Spleen		Kidney	
		mg/g dry tissue	*mg in total liver/100 g body wt*	*mg/g dry tissue*	*mg in total spleen/100 g body wt*	*mg/g dry tissue*	*mg in total kidney/100 g body wt*
Iron	normal	0.63 ± 0.07 [1]	0.86 ± 0.54	3.31 ± 0.54	0.10 ± 0.02	0.42 ± 0.04	0.07 ± 0.01
	treated	0.71 ± 0.04	0.93 ± 0.06	3.12 ± 0.18	0.13 ± 0.02	0.43 ± 0.03	0.09 ± 0.01
Magnesium	normal	0.91 ± 0.04	1.20 ± 0.04	1.19 ± 0.06	0.044 ± 0.002	0.99 ± 0.02	0.20 ± 0.01
	treated	0.99 ± 0.04	1.30 ± 0.09	1.18 ± 0.06	0.041 ± 0.003	0.96 ± 0.02	0.20 ± 0.01
Calcium	normal	0.73 ± 0.10	0.95 ± 0.08	1.01 ± 0.07	0.039 ± 0.001	1.34 ± 0.10	0.32 ± 0.07
	treated	0.60 ± 0.06	0.81 ± 0.05	0.85 ± 0.07	0.035 ± 0.003	1.39 ± 0.12	0.28 ± 0.03
Phosphorus	normal	11.25 ± 0.30	14.30 ± 0.70	16.96 ± 0.94	0.56 ± 0.02	12.69 ± 0.33	2.48 ± 0.10
	treated	11.69 ± 0.49	14.62 ± 0.40	15.14 ± 0.80	0.63 ± 0.03	12.40 ± 0.27	2.52 ± 0.10

[1] SE of the mean.

urinary excretion is due to a decreased tubular reabsorption of calcium and phosphate.

The significant increase of urinary calcium in patients with Wilson's disease (9), and of urinary calcium and phosphorus following oral administration of penicillamine to guinea pigs, suggests that special attention should be given to meeting the nutritional requirements for calcium and phosphorus.

LITERATURE CITED

1. Walshe, J. M. 1964 Endogenous copper clearance in Wilson's disease: a study of the mode of action of penicillamine. Clin. Sci., 26: 461.
2. Osborn, S. B., and J. M. Walshe 1958 Effects of penicillamine and dimercaprol on turnover of copper in patients with Wilson's disease. Lancet, *1:* 70.
3. Djafar, M. I., H. R. Camberos and G. K. Davis 1968 Copper limitation with penicillamine. J. Florida Acad. Sci., in press.
4. Walshe, J. M. 1956 Penicillamine, new oral therapy for Wilson's disease. Amer. J. Med., *21:* 487.
5. Goldstein, N. P., R. V. Randall, J. B. Gross, J. W. Rosevear and W. F. McGuckin 1962 Treatment of Wilson's disease (hepatolenticular degeneration). Neurology, *12:* 231.
6. Lenz, G. R., and A. E. Martell 1964 Metal chelates of some sulfur containing amino acids. Biochemistry, 3: 745.
7. Harris, C. E. C. 1958 A comparison of intravenous calcium disodium versenate and oral penicillamine in promoting elimination of lead. Can. Med. Assoc. J., *79:* 664.
8. Aposhian, H. V. 1958 Protection by d-penicillamine against the lethal effects of mercuric chloride. Science, *128:* 93.
9. Litin, R. B., N. P. Goldstein, R. V. Randall and G. R. Diessner 1958 Hypercalciuria in Wilson's disease with data on the effect of dl-penicillamine on the urinary excretion of calcium and copper. J. Lab. Clin. Med., 52: 923.
10. Litin, R. B., N. P. Goldstein, R. V. Randall, M. H. Powers and G. R. Diessner 1960 Effect of D,L-penicillamine in the urinary excretion of copper and calcium in hepatolenticular degeneration (Wilson's disease). Neurology, *10:* 123.
11. Boulding, J. E., and R. A. Baker 1957 The treatment of metal poisoning with penicillamine. Lancet, 2: 985.
12. Fiske, C. A., and Y. Subbarow 1925 The colorimetric determination of phosphorus. J. Biol. Chem., 66: 375.
13. Snedecor, G. W. 1956 Statistical Methods, ed. 5. Iowa State University Press, Ames.
14. Keiser, H. R., R. I. Henkin, F. C. Bartter and A. Sjoerdsma 1968 Loss of appetite during therapy with penicillamine. J. Amer. Med. Assoc., 203: 381.

15. Sternlieb, I., and I. H. Scheinberg 1964 Penicillamine therapy for hepatolenticular degeneration. J. Amer. Med. Assoc., *189:* 748.
16. Chang, Y. C., C. C. Ch'in and H. W. Tsou 1957 Clinical observations on twenty-five cases of hepatolenticular degeneration. Chin. Med. J., *75:* 516.
17. Feng, Y. K. 1957 Wilson's disease — report of ten cases. Chin. Med. J., *75:* 631.
18. Finbay, N., and A. G. Bearn 1958 Roentgenographic abnormalities of the skeletal systems in Wilson's disease. Amer. J. Roentgenol., *79:* 603.
19. Randall, R. V., N. P. Goldstein, J. B. Gross and J. W. Rosevear 1966 Hypercalciuria in hepatolenticular degeneration. Amer. J. Med. Sci., *252:* 715.
20. Playoust, M. R., and N. E. Dale 1961 Metabolic balance studies in two patients with Wilson's disease and hypercalciuria. Metabolism, *10:* 304.
21. Morgan, H. G., W. K. Stewart, K. G. Lowe, J. M. Stowers and J. H. Johnstone 1962 Wilson's disease and the Fanconi syndrome. Quart. J. Med., *31:* 361.
22. Lutwak, L. 1964 Myositis ossificans progressiva. Amer. J. Med., *37:* 269.

Whole-body Retention, Tissue Distribution and Excretion of Selenium-75 After Oral and Intravenous Administration in Lambs Fed Varying Selenium Intakes [1,2]

PERLA L. LOPEZ, R. L. PRESTON AND W. H. PFANDER
Department of Animal Husbandry, University of Missouri, Columbia, Missouri

ABSTRACT Selenium metabolism was studied by measuring whole-body retention, urine and fecal losses and retention in selected tissues following administration of ^{75}Se to lambs fed varying levels of dietary selenium. Whole-body loss of ^{75}Se 48 to 336 hours after administration of the isotope could be described by a first-order rate constant which was inversely proportional to the dietary level of selenium. Intravenous injection of ^{75}Se resulted in a higher retention than oral administration, especially on low dietary intakes of selenium. The concentration of ^{75}Se in various tissues was inversely related to the dietary selenium level. Kidney and liver tissue were consistently higher in ^{75}Se concentration than any other internal organ tissue. Of the total ^{75}Se dose administered, the carcass retained the highest amount, being indirectly proportional to the dietary selenium level. The major pathways of ^{75}Se excretion are the feces and urine. The kidney exhibited an unlimited ability to excrete ^{75}Se. Since a significant amount of ^{75}Se excretion was not accounted for as urine and fecal loss, respiratory loss of volatile selenium products formed in the rumen is offered as a possible explanation. Relative importance of these three excretory pathways depended upon dietary selenium intake level and route of ^{75}Se administration.

In recent years, a number of investigators have studied the distribution and elimination of selenium in several species. Studies with rats are numerous (1–5); however, ruminant whole-body turnover studies using ^{75}Se have not been reported, although this isotope has been used to study certain aspects of selenium metabolism in sheep (6–11). In vivo turnover of ^{75}Se in rats was studied by Blincoe (12).

The present studies were carried out to determine whole-body retention, tissue distribution and excretion of ^{75}Se in lambs maintained for at least 6 weeks on varying dietary levels of selenium.

A spectrometer designed for whole-body counting was used in this work. This technique has the advantage of measuring in vivo or in situ without destroying the animal to obtain a sample for assay. Due to the limited number of "whole-body counters," studies on mineral metabolism using this technique are limited.

EXPERIMENTAL

Experimental animals and rations used in these studies were described in a previous report (13). The basal diet was composed of: (in percent) Torula yeast (6.5), Solka Floc (BW-40),[3] (30), corn starch (30), glucose (23.5), urea (3), lard (3) and minerals and vitamins. Selenium as Na_2SeO_3 was added to this basal diet to achieve the desired levels.

Experiment 1. After receiving diets containing 0.016, 0.516, 1.016, 1.516 and 5.016 ppm Se as well as a natural ration, for 97 days, the lambs were transferred to metabolism cages for separate quantitative collection of feces and urine. Each lamb was given an oral dose of ^{75}Se (1.5 μCi) as Na_2SeO_3 in a gelatin capsule. Each capsule was counted in a gamma spectrometer [4] with a 12.7 cm by 12.7 cm sodium iodide (T1) detector.

Received for publication May 31, 1968.

[1] Contribution from the Missouri Agricultural Experiment Station. Journal Series no. 5410; approved by the Director. Supported jointly by funds provided by the Missouri Agricultural Experiment Station and a Rockefeller Foundation Fellowship.

[2] Part of doctoral dissertation of the senior author, Department of Animal Husbandry, University of Missouri, Columbia, Missouri 65201.

[3] Solka Floc (BW-40), Brown Company, Berlin, N. H.

[4] Model 34-12, Radiation Instrument Development Laboratory, Inc., Melrose Park, Ill.

Total urine and feces were collected at 24-hour intervals. Feces were weighed and placed in plastic bags. Urine volumes were measured and a 50-ml aliquot from each urine sample was placed in a polyethylene bottle. Each sample was counted for ^{75}Se content in the same counter used for the capsules.

The lambs were killed on day 8 after dosing with ^{75}Se. Brain, lung, heart, liver, kidney, spleen, adrenal, gastrocnemius muscle, a section of the dorsal sac of the rumen, a portion of small and large intestine, cecum and hide were collected. The different tissues were stored at $-20°$ until counted for ^{75}Se.

Experiment 2. After the lambs were fed 0.014, 0.264, 0.514 and 5.014 ppm Se for 75 days, they were individually housed in metabolism cages for separate collection of feces and urine. Two lambs on each treatment were given an oral dose of ^{75}Se via capsules as in experiment 1. Each capsule contained 2 μCi of ^{75}Se. All capsules were counted twice in a liquid scintillation whole-body counter[5] and the average count of the eight capsules was used as initial dose given to each lamb receiving ^{75}Se orally. The two other lambs in each dietary group were given an intravenous dose of ^{75}Se (2 μCi) by infusion into the left jugular vein. This same procedure was repeated four times; in each case, the dose and saline washings were received in polyethylene bottles which were counted in the whole-body counter. The average count for these four bottles was used as the intravenous dose of ^{75}Se administered to each animal.

Each lamb was counted before dosing to determine the background count; whole-body count was then determined within 5 to 10 minutes after administration of ^{75}Se and at the following subsequent time intervals, 6, 12, 24, 48, 96, 144, 192, 240, 288 and 336 hours. Total urine and feces voided between each counting period were collected. Feces were weighed and kept in plastic bags. Urine volumes were recorded and a 250-ml aliquot was collected in a polyethylene bottle.

Half of the experimental animals were killed on day 12 and the rest on day 15, after dosing with ^{75}Se. Total blood from each animal was collected in plastic pails. Selected tissues were collected and placed in separate plastic bags as follows: pelt, head and hoofs, gastrointestinal (GI) tract, whole carcass, heart, lung, liver, bile (gallbladder), spleen, kidney, adrenal and esophagus. Some of the organs were studied further, after each was counted. The brain was counted separately from the head. The gastrointestinal tract was separated into the four stomachs (reticulum, rumen, omasum and abomasum), small intestines, cecum and large intestines. Each separate organ was counted with and without its contents. Adipose tissue from the abdominal cavity was also counted. The soft flesh from one-half of the carcass was separated from the bone, and each was counted for ^{75}Se content. The kidneys were dissected and the medulla removed from the cortex; each was counted for radioactivity. The weights of all tissues and organs were recorded.

Tissue, fecal and urine samples were carefully wrapped in plastic bags and were counted using the same geometry as the capsules. Live animals and carcasses were placed on a cart and were covered with cotton cloth before counting in the whole-body counter.

The whole-body counter had three pairs of scintillation tanks. Each tank contained a photomultiplier tube and 23 gal of scintillation liquid consisting of 2,5-diphenyloxazole (PPO) and 2,2′ paraphenylene bis 5-phenyloxazole (POPOP) in toluene as the primary and secondary fluor, respectively. During this experiment only the center pair of tanks was used. The counter was preset such that pulses with an amplitude less than 250 and greater than 1800 kev did not appear in the analyzer output. At this setting 99.4% of the gamma counts from ^{75}Se were detected in the low energy channel (250 to 400 kev). Efficiency for the geometry used in counting the capsules was 8.32%. All counts were duplicate 1-minute counts.

All calculations on whole-body retention, tissue distribution and excretion of ^{75}Se are corrected for decay of the isotope.

RESULTS

Whole body retention. Exp. 2. There was considerable variation in whole-body

[5] Packard Instrument Co., Inc., Downers Grove, Ill.

count determined 5 to 10 minutes through 24 hours after ^{75}Se administration, regardless of selenium level in the diet and mode of isotope administration. Whole-body counts decreased constantly in all lambs beyond 24 hours. Therefore, data from 48 hours through the end of the retention study were fitted to a linear regression, using a log transformation of the whole-body counting rates. A high linear correlation ($r = 0.90$ to 1.00) resulted (table 1); however, extrapolation of each linear equation to zero time gave significantly different ($P < 0.01$) a values (gamma counts at zero time) between lambs. A wide variation in a values seemed improb-

TABLE 1

Values for intercept, regression coefficient and correlation coefficient of the linear regression equations ($\log y = a + bx$) on whole-body retention of ^{75}Se by individual lambs [1]

Dietary selenium level	Route of administration	Replicate	a	b	r
ppm				$\times 10^{-3}$	
0.014	Oral	1	4.8160	−0.5891	0.98
		2 [2]	—	—	—
	Intravenous	1	5.0238	−0.2896	0.90
		2	5.0525	−0.2855	0.90
0.264	Oral	1	4.7985	−1.5050	0.98
		2	4.8560	−1.6670	0.98
	Intravenous	1	4.8704	−0.7767	1.00
		2	4.8478	−0.7487	0.99
0.514	Oral	1	4.6955	−1.7800	0.99
		2	4.9292	−2.2250	0.99
	Intravenous	1	4.8811	−1.3890	0.99
		2	4.7674	−1.4390	0.99
5.014	Oral	1	4.5988	−4.0260	0.98
		2	4.8038	−3.6900	0.98
	Intravenous	1	4.4848	−2.2890	0.99
		2	4.5654	−1.9170	0.98

[1] Data from 48 to 336 hours were used for regression analyses (y = cpm; x = hours after ^{75}Se administration).
[2] Denotes lamb was lost.

TABLE 2

The a, b and c values of the second-degree equations ($\log y = a + bx + cx^2$) on whole-body retention of ^{75}Se by individual lambs [1]

Dietary selenium level	Route of administration	Replicate	a	b	c
ppm				$\times 10^{-3}$	$\times 10^{-6}$
0.014	Oral	1	4.9455	−2.1306	3.6707
		2 [2]	—	—	—
	Intravenous	1	5.0242	−0.1845	0.3953
		2	5.0099	−0.2595	1.4855
0.264	Oral	1	4.9566	−3.4740	4.8455
		2	4.9444	−3.1450	4.4846
	Intravenous	1	4.9143	−1.2214	0.9873
		2	4.9084	−1.6375	2.5510
0.514	Oral	1	4.8713	−3.8435	4.9486
		2	4.9826	−3.2200	3.5380
	Intravenous	1	4.9666	−2.3120	2.1021
		2	4.8941	−3.1281	4.9210
5.014	Oral	1	4.9760	−8.9535	12.4770
		2	5.1283	−8.6395	14.5200
	Intravenous	1	4.8470	−6.7123	10.8130
		2	4.8852	−6.3240	12.2930

[1] Data from zero to 336 hours were used for regression analyses (y = cpm; x = hours after ^{75}Se administration).
[2] Denotes lamb was lost.

able since extrapolated values were not related to either size of the animal or route of ^{75}Se administration. Because a curvilinear relation may exist between the log of the whole-body count and time after ^{75}Se administration, data from zero time were fitted to a second-degree equation (table 2). The *a* values of each quadratic equation were not significantly different between individual lambs, dietary selenium levels or mode of ^{75}Se administration.

Retention curves, represented by a quadratic equation which is the average of the two equations for lambs that received the same treatment, show that whole-body retention of ^{75}Se is a function of dietary selenium intake and route of selenium administration. The *b* values obtained, either by quadratic or linear regression analyses, showed the marked effect of dietary selenium level and mode of ^{75}Se administration ($P < 0.001$).

The biological half-life ($T_{1/2}B$) of selenium based on whole-body loss 48 to 336 hours after oral administration of ^{75}Se was 636, 202, 162 and 82 hours for the 0.01, 0.26, 0.51 and 5.01 ppm Se levels, respectively. Similarly, the half-life of intravenously administered ^{75}Se was 1660, 469, 229 and 149 hours, respectively. The effective half-life ($T_{1/2}E$), which is the combined effect of biological half-life and radiological half-life ($T_{1/2}P$) of ^{75}Se, was calculated with the formula:

$$T_{1/2}E = \frac{T_{1/2}P + T_{1/2}B}{T_{1/2}P\ T_{1/2}B}$$

The calculated $T_{1/2}E$ was 521, 189, 154 and 80 hours for the oral dose and 1048, 402, 212 and 142 hours for the infused dose of ^{75}Se for lambs on the 0.01, 0.26, 0.51 and 5.01 ppm Se, respectively.

Fecal, urinary and respiratory excretions of ^{75}Se. Exp. 1. Fecal excretion of ^{75}Se of all lambs was maximum 48 hours after isotope administration, decreased rapidly during the next 48 hours, and decreased slowly through the remainder of the excretion study. Urinary excretion exhibited a similar trend; a rapid drop in ^{75}Se excretion was observed for all lambs at 72 hours, followed by an almost constant decrease throughout the 8-day observation period.

Dietary selenium levels were reflected in urinary excretion but not in fecal excretion of ^{75}Se; excretion in the urine during the 8-day period increased with increasing dietary selenium level. These differences in urinary excretion of lambs on different dietary treatments were observed within 48 hours after ^{75}Se administration.

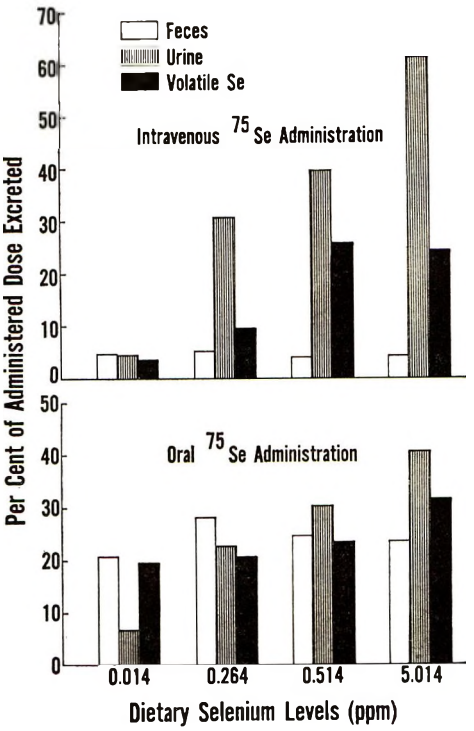


Fig. 1 Excretion of ^{75}Se by lambs fed varying selenium intakes after administration of the isotope during a 12-day period.

Experiment 2. The elimination of ^{75}Se in the feces, urine and in the calculated volatile form is presented in figure 1. Volatile selenium was calculated as the difference of 100 minus the whole-body retention of ^{75}Se and total fecal and urinary excretions on a percentage basis. Whole-body retention was calculated from body count of the animal on day 12 after selenium administration, divided by the estimated *a* value (counts at zero time) of the second-degree equation. Fecal and urinary excretions were computed as percentage of the activity of the capsule or of the infused ^{75}Se. Excretion values were not corrected for fecal and urinary loss when the lambs were counted and in transit (approximately 100 m distance). A conservative estimate of this loss would be 10%

of the total fecal and urinary excretion. Thus, the computed value for volatile selenium compounds is an overestimate and also includes all possible analytical errors.

The excretion pattern of ^{75}Se was different between intravenous and oral administration of the isotope. Volatile elimination by lambs on 0.01 ppm Se which received the isotope orally was slightly lower than the fecal loss but higher than the urinary excretion; this pattern was reversed in lambs on 5.01 ppm Se. Volatile loss was slightly lower than fecal and urinary excretion of lambs on 0.26 and 0.51 ppm levels. Fecal excretion was higher than urinary loss in lambs on 0.01 and 0.26 ppm levels. The reverse was observed in the higher dietary groups (0.51 and 5.01 ppm Se). Respiratory excretion was higher than fecal in lambs infused with ^{75}Se but lower than urinary excretions in all dietary groups, except the 0.01 ppm level. Radioactive selenium in the feces and urine was about the same and was slightly higher than volatile losses in lambs fed the basal diet. Urinary and volatile excretion increased with increasing dietary selenium level, especially when the isotope was intravenously administered.

Respiratory and urinary ^{75}Se excretion was dependent ($P < 0.05$) both on dietary selenium levels and route of ^{75}Se administration; however, route of isotope administration is the major factor affecting fecal loss of ^{75}Se. The effect of dietary selenium intake on urinary excretion of ^{75}Se was observed within 48 hours after isotope administration; the difference in fecal excretion relative to route of administration was apparent within 24 hours.

Fecal and urinary ^{75}Se excretions were maximum at 96 hours regardless of dietary selenium level and route of administration with few exceptions. Lambs on the 5.01 ppm level that received ^{75}Se intravenously had maximum activity of the isotope in the urine within 12 hours after administration. In contrast, lambs on 0.01 and 0.26 ppm levels that received oral ^{75}Se had maximum concentration of the isotope in the urine between 96 and 144 hours after ^{75}Se administration. Lambs on this same dietary selenium level which received an intravenous dose of ^{75}Se exhibited a maximum fecal excretion between 144 and 192 hours after isotope administration. A constant decrease followed after maximum excretion in all dietary groups with either mode of administration.

Distribution of retained ^{75}Se in tissues. Exp. 1. Selenium-75 retained per gram of tissue decreased with increasing dietary level of selenium. The greatest amount of ^{75}Se in the internal organs was found in the liver, kidney and lungs when results were based on the amount of ^{75}Se in the whole organ; the liver retained the highest amount in lambs fed the diets with added selenium, whereas the kidney retained the greatest amount in the lamb fed the basal diet.

When results were based on ^{75}Se concentration, retention in descending order was as follows: kidney, adrenal, liver, spleen, thyroid, lung, bile (gallbladder), brain and heart. Accumulations of ^{75}Se in the pelt, gastrocnemius muscle and femur bone were about the same and were higher than in adipose tissue. Concentration of ^{75}Se in the small intestines was higher than in either the rumen or large intestines.

Experiment 2. The distribution of ^{75}Se in the tissues of animals fed varying levels of selenium and killed on day 12 and day 15 after oral and intravenous administration of radioactive selenium are shown in figures 2 through 4.

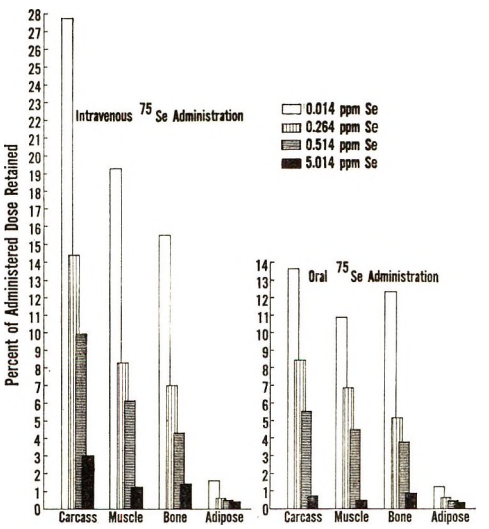


Fig. 2 Average distribution of ^{75}Se in carcass and constituent parts of lambs fed varying selenium intakes.

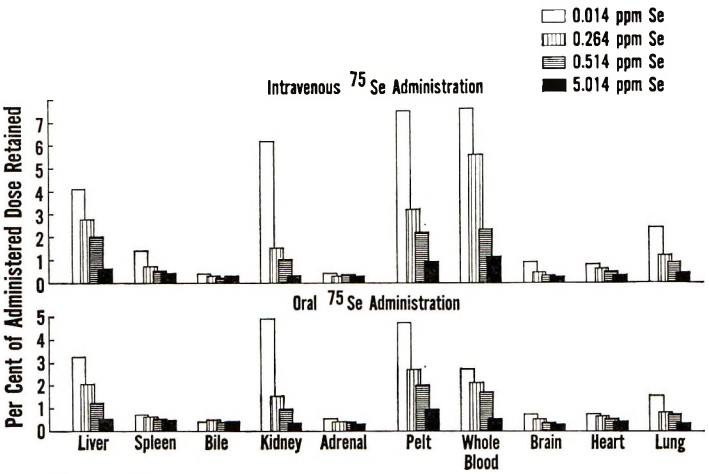


Fig. 3 Average distribution of retained ^{75}Se in tissues of lambs fed varying selenium intakes.

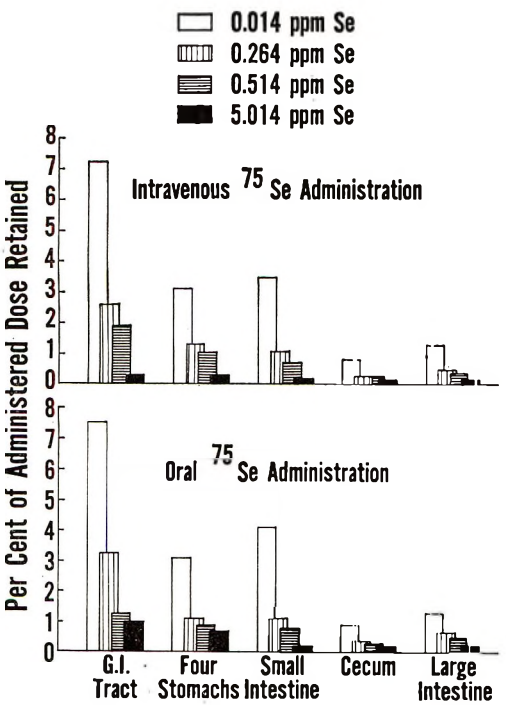


Fig. 4 Average distribution of retained ^{75}Se in gastrointestinal tract including contents of lambs fed varying selenium intakes.

Retention of ^{75}Se in the tissues was calculated as a percentage of the activity of the capsule or of the infused dose. Retained ^{75}Se in the carcass was determined as a percentage of the whole-body count at zero time (*a* value, second-degree equation).

Selenium levels in the diet showed a marked influence on the ^{75}Se concentration retained by the tissues. Tissue retention of ^{75}Se decreased with increasing dietary selenium level ($P < 0.01$). A higher ^{75}Se retention in the tissues was observed in lambs that received the intravenous dose ($P < 0.01$ to 0.05) with some exceptions. The ^{75}Se retention in tissues of lambs killed on days 12 and 15 were generally not statistically different.

The carcass retained the greatest amount of ^{75}Se. Activity in the carcass was distributed between the muscles and bones in approximately equal amounts. A negligible amount of the isotope was present in adipose tissue. Carcasses of lambs killed on day 12 after isotope administration contained a greater amount of ^{75}Se ($P < 0.05$) than those killed on day 15 regardless of mode of administration; however, the difference was not significant when either the muscle, bone or adipose tissues were examined separately.

The internal organs retained a higher concentration of activity than other tissues when the results were expressed per gram tissue. Lambs fed the basal ration retained higher ^{75}Se concentration in the kidney than in the liver, irrespective of mode of administration. Retention by these tissues was reversed in lambs that received added

selenium in the ration. More than 99% of the activity in the kidney was present in the cortex; a negligible amount was present in the medulla.

The order of ^{75}Se distribution in the various parts of the gastrointestinal tract of lambs receiving the basal ration was the small intestines > four stomachs > large intestines > cecum regardless of mode of ^{75}Se administration. The same trend was true for lambs that received added selenium in the diet except that the four stomachs were higher in ^{75}Se concentration than in the small intestines. The relative distribution of retained ^{75}Se in these tissues with respect to dietary selenium intake did not change when the contents were washed from the organs. The four stomachs free of contents, from lambs that received an intravenous dose of ^{75}Se, were higher ($P < 0.05$) in activity than those given the oral ^{75}Se. The activity of the washed small intestine was not affected by mode of administration. In contrast, the large intestines and cecum when washed retained more ^{75}Se after an intravenous dose than after oral administration ($P < 0.01$).

When the results were compared per gram tissue, the adrenal, kidney, bile, spleen, liver, brain, lungs, heart, whole blood, pelt, gastrointestinal tract and carcass in decreasing order retained the highest concentration of ^{75}Se. There were some variations in the amount of retained ^{75}Se in the different tissues as influenced by dietary selenium but the maximum concentration of ^{75}Se occurred in the tissues of lambs that received the basal diet regardless of mode of ^{75}Se administration.

DISCUSSION

It appears from the quadratic curves that whole-body turnover of ^{75}Se in lambs on varying selenium intake may be described by two first-order processes with different rate constants, the initial slope being greater than the final although the final slope increased with increasing selenium intake irrespective of mode of ^{75}Se administration. Whether the initial slope is actually another rate constant or merely represents the total unequilibrated selenium is difficult to evaluate. The relatively short period (48 hours) covered by the initial slope which corresponds to maximum selenium excretion rates would indicate that this part of the quadratic curve represents the total unequilibrated selenium, consisting of that not absorbed from the gastrointestinal tract and the absorbed selenium not equilibrated in the tissues. The unabsorbed selenium is eliminated in the feces whereas the latter is excreted in the urine, the expired air or reabsorbed into the gastrointestinal tract following biliary excretion.

Results of the present study show conclusive evidence that selenium undergoes active metabolism in the body of the animal. The metabolic rate is governed by a first-order process where the rate constant varied according to dietary intake and mode of administration over a period of 12 to 15 days. This may indicate that the rate limiting process is the release of selenium from the various tissues in which it is bound. Based on the b values of the linear regressions (table 1), selenium supplementation in lambs with low selenium intake would be more beneficial if administered intravenously than orally, since the lambs would retain twice as much selenium by this route of administration. Route of selenium administration, however, showed no marked differences in body retention at dietary levels of 0.51 ppm Se or higher.

The effective half-life of ^{75}Se in lambs decreased with increasing selenium level in the diet. The rate of decrease was not linear suggesting that at higher dietary levels (> 5 ppm) selenium turnover may not be further increased. This same trend was observed in the study of in vitro uptake of ^{75}Se by ovine blood cells of lambs in the same dietary groups (13). Blincoe (12) obtained a retention curve for rats similar to the quadratic curves observed in this study.

Several authors (7–10, 14, 15) have compared selenium excretion in the feces and urine of sheep. Several studies on respiratory elimination of selenium in rats have also been reported (1–4, 16). It is evident from this study that selenium excretion by any one pathway is dependent on the dietary selenium intake and route of ^{75}Se administration.

Generally, respiratory excretion increased with increasing selenium intake

irrespective of route of ^{75}Se administration. This is in agreement with the results obtained on rats by Ganther et al. (4). They reported that volatilization was increased greatly from a purified diet containing 0.03 ppm Se to a crude commercial diet with 0.5 ppm of the element.

Considerable evidence indicates that dimethylselenide is the metabolic product of inorganic selenium metabolism in several biological processes (4, 17). The volatile product is normally synthesized in the liver, and transported by the blood to the lungs. In our study, formation of volatile selenium compounds was higher following the oral dose than the intravenous dose. The difference between the two routes was greater on the lower dietary levels of selenium (0.01 and 0.26 ppm); on the higher levels (0.51 and 5.01 ppm), volatilization of selenium from the oral dose was only slightly higher than the intravenous dose. Since lambs on lower selenium intake which were dosed orally with ^{75}Se volatilized a greater amount of selenium than lambs dosed intravenously, formation of volatile or gaseous selenium products in the gastrointestinal tract, possibly in the rumen, is suggested.

Selenium as sodium selenite (Na_2SeO_3) is in the + 4 oxidation state. Reduction of Na_2SeO_3 could readily occur in the rumen due to its anaerobic, highly reducing atmosphere. Rosenfeld and Beath (18) reported that bacteria are capable of reducing selenium salts to elemental selenium which in turn may form metal selenides. Metal selenides on hydrolysis form hydrogen selenide (H_2Se), a colorless, very toxic gas closely resembling H_2S in general properties. There is evidence that small amounts of H_2S are formed in the rumen. The analogy of selenium to sulfur in chemical behavior makes it reasonable to postulate the formation of H_2Se in the rumen.

Dimethylselenide could be formed in the rumen from metal selenide and methyl compounds. The difference in volatilization between oral and intravenous administration on the lower dietary selenium groups suggests that a greater proportion of metal selenides are formed into dimethylselenide than into H_2Se due to the highly toxic nature of the latter. Dimethylselenide may be absorbed into the blood and eliminated through the respiratory tract together with that formed in the liver. Hydrogen selenide may be eliminated together with some of the gases from the rumen via eructation.

Respiratory excretion in lambs given ^{75}Se intravenously was the same for the 0.51 and 5.01 ppm Se levels. This suggests that there is a limit in the synthesis of dimethylselenide by the liver, resulting in an increase in urinary excretion of the isotope. Lambs that received ^{75}Se by capsule, however, showed a further increase in respiratory loss above the 0.51 ppm level. This difference in respiratory elimination between intravenous and oral administration of the isotope further supports the hypothesis that volatile selenium products are formed in the rumen.

Urinary excretion of ^{75}Se increased markedly with increasing selenium level in the diet, suggesting that at these dietary levels there is no limit to the amount of ^{75}Se that can be eliminated by this pathway.

Selenium-75 in the feces was essentially the same in all dietary groups receiving the isotope orally. This suggests that total absorption of ingested selenium from the gastrointestinal tract is a direct function of selenium intake of the animal. On the other hand, equal fecal excretions in all dietary groups that received ^{75}Se intravenously suggest that ^{75}Se secretion from the body into the lumen of the gastrointestinal tract is constant and not related to dietary selenium level.

Wright (10) obtained a higher concentration of ^{75}Se in the urine than in the feces of ewes fed on hay after intravenous injection of the isotope. Peterson and Spedding (8) and Butler and Peterson (6) reported that selenium is excreted predominantly in the feces of sheep fed on red clover grown in ^{75}Se solution. Rosenfeld and Eppson (9) observed in sheep with chronic selenosis that urinary excretion was slightly higher than fecal elimination. These results are in general agreement with those presented in this study and indicate that the major route of selenium excretion is a function of the present selenium status of the animal. Other factors such as nature of the diet and mode of selenium administration also may affect

the route of excretion. It is difficult, however, to accept the statements made by Butler and Peterson (6) and Cousins and Cairney (7) that the major excretory pathway in sheep is via the feces.

Of the ^{75}Se retained by the lamb, the carcass contained the greatest amount of all tissues examined. The sum of retained ^{75}Se in the muscle, bone and adipose tissues was higher than that of the carcass. This discrepancy is probably explained by the difference in geometry of counting. Selenium-75 in the carcass was distributed between the muscles and bones in approximately equal amounts, with an almost negligible amount in the adipose tissue. This suggests that selenium is associated with tissue proteins and these tissues are adept in binding selenium with the proteins. These results support those of Rosenfeld (3) and Hopkins et al. (5) that the carcass of rats retained the highest concentration of selenium when results were based on the whole body, irrespective of dietary selenium levels and route of administration.

The liver, kidney and lung retained the highest concentration of ^{75}Se of the internal organs and tissue fluids, irrespective of dietary selenium levels or mode of administration. This conforms with the results of Rosenfeld and Eppson (9) and Wright (11). Studies on tissue distribution of ^{75}Se in other species such as mice, rats, dogs and swine are similar with the findings in these experiments (3, 5, 19). This suggests that in the liver, kidney and lung, selenium exchange with ^{75}Se in the plasma is more rapid and complete, whereas in the brain, heart, spleen, bile and adrenal, selenium is exchanged more slowly.

Lambs fed the basal ration retained a greater amount of selenium in the kidney with both modes of administration. This was also observed in experiment 1 in lambs fed similar levels of dietary selenium. This was not expected since the urine was not the main excretory pathway in lambs on low selenium intake. This may suggest some specific function of the kidney in selenium metabolism other than excretion.

Considerable concentration of ^{75}Se was found in the pelt (fig. 3). It is possible that part of this radioactivity is due to ^{75}Se excreted via the skin. Previous reports show that selenium is deposited and retained in the hide, wool and hair of animals (14, 20–22).

ACKNOWLEDGMENTS

The authors thank Dr. E. R. Graham for helpful suggestions relative to whole-body counting of the animals, and W. Coffman for technical assistance.

LITERATURE CITED

1. McConnell, K. P., and O. W. Portman 1952 Excretion of dimethylselenide by the rat. J. Biol. Chem., *195:* 277.
2. Olson, O. E., B. M. Schulte, E. T. Whitehead and A. W. Halverson 1963 Effects of arsenic on selenium metabolism in rats. J. Agr. Food Chem., *11:* 531.
3. Rosenfeld, I. 1964 Metabolic effects and metabolism of selenium in animals. IV. Excretion and retention of ^{75}Se in relation to modes of administration, toxicity and pregnancy in rats. Wyoming Agr. Exp. Sta. Bull. no. 414, p. 35.
4. Ganther, H. E., O. A. Levander and C. A. Baumann 1966 Dietary control of selenium volatilization in the rat. J. Nutr., *88:* 55.
5. Hopkins, L. L., Jr., A. L. Pope and C. A. Baumann 1966 Distribution of microgram quantities of selenium in the tissues of the rats and effects of previous selenium intake. J. Nutr., *88:* 61.
6. Butler, G. W., and P. J. Peterson 1961 Aspects of the fecal excretion of selenium by sheep. New Zeal. J. Agr. Res., *4:* 484.
7. Cousins, F. B., and I. M. Cairney 1961 Some aspects of selenium metabolism in sheep. Australian J. Agr. Res., *12:* 927.
8. Peterson, P. J., and D. J. Spedding 1963 The excretion by sheep of ^{75}Se incorporated into red clover (*Trifolium pratense* L.): The chemical nature of selenium and its uptake by three plant species. New Zeal. J. Agr. Res., 6: 13.
9. Rosenfeld, I., and H. F. Eppson 1964 Metabolic effects and metabolism of selenium in animals. V. Metabolism of selenium in sheep. Wyoming Agr. Exp. Sta. Bull. no. 414, p. 53.
10. Wright, E. 1965 The distribution and excretion of radioselenium in sheep. New Zeal. J. Agr. Res., 8 (2): 284.
11. Wright, E. 1965 The concentration of radioselenium by the tissues of selenium-responsive sheep in relation to their growth rates. New Zeal. J. Agr. Res., 8 (2): 292.
12. Blincoe, C. 1960 Whole-body turnover of selenium in rats. Nature, *186:* 398.
13. Lopez, P. L., R. L. Preston and W. H. Pfander 1968 In vitro uptake of selenium-75 by red blood cells from the immature ovine during varying selenium intakes. J. Nutr., *94:* 219.

14. Rosenfeld, I., and O. A. Beath 1945 The elimination and distribution of selenium in the tissues in experimental selenium poisoning. J. Nutr., *30:* 443.
15. Butler, G. W., and P. J. Peterson 1963 Availability of selenium in forage to ruminants. Proc. New Zeal. Soc. Anim. Prod., *23:* 13.
16. Schultz, J., and H. B. Lewis 1940 The excretion of volatile selenium compounds after administration of sodium selenite to white rats. J. Biol. Chem., *133:* 199.
17. McConnell, K. P., and B. J. Cooper 1950 Distribution of selenium in serum proteins and red blood cells after subcutaneous injection of sodium selenate containing radioselenium. J. Biol. Chem., *183:* 459.
18. Rosenfeld, I., and O. A. Beath 1964 Selenium: Geobotany, Biochemistry, Toxicity and Nutrition. Academic Press, New York.
19. Lindberg, P., and M. Siren 1963 Selenium concentration in kidneys of normal pigs and pigs affected with nutritional muscular dystrophy and liver dystrophy. Life Sci., *5:* 326.
20. Demiruren, A. S., and B. S. Alen 1963 Effects of selenium on certain mechanical properties of the wool fiber. Can. J. Anim. Sci., *42* (2): 233.
21. McConnell, K. P., and A. E. Kreamer 1960 Incorporation of ^{75}Se with dog's hair. Proc. Soc. Exp. Biol. Med., *105:* 170.
22. Underwood, E. J. 1962 Trace Elements in Human and Animal Nutrition, ed. 2. Academic Press, New York.

Purified Diet for Dental Caries Research with Rats [1]

JUAN M. NAVIA, HADY LOPEZ AND ROBERT S. HARRIS
Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts

ABSTRACT A new caries-promoting diet (MIT-200, percentage composition of purified ingredients: pulverized sucrose, 67; lactalbumin, 20; MIT salt mixture, 3; vitamin mixture, 1; cottonseed oil, 3; and cellulose, 6) was formulated and tested for usefulness in a caries research experiment with rats. It was compared with a previously used caries-promoting diet (MIT-10) composed of natural foods in terms of rat body weight gain and types and severity of molar lesions after either diet had been fed for 15 days prenatally, 65 days postnatally, or both. Effects of these diets upon responses to a cariostatic agent (0.5% sodium trimetaphosphate) were also evaluated. Rats fed MIT-200 showed higher caries scores on all molar surfaces, than those fed MIT-10, but the lesions did not penetrate as far into the dentin. Sodium trimetaphosphate was significantly more effective in controlling caries when fed in MIT-200 than in MIT-10. Its primary effect was to reduce the extent of caries penetration rather than to influence the initiation of the caries attack. Diet MIT-200 is superior to natural food diets for dental caries research with rats because its composition is reproducible, it is adequate nutritionally, it permits the development of all types of caries lesion, and it can be used for the assay of cariostatic agents.

Precise studies of the effects of specific minerals upon the development of experimental caries cannot be conducted unless the mineral content of the animal environment (cage, water, diet, atmosphere) are under strict control. Caries-producing diets composed of natural foodstuffs such as cereals, legumes, alfalfa and milk powder, are not fully satisfactory for trace-element research because the amounts and types of mineral complexes normally present in these foods vary from batch to batch and cannot be standardized.

Several investigators (1–9) have used purified diets in experimental caries research. Some of these diets are nutritionally inadequate for the experimental animal involved, and some contain liver powder, yeast powder or alfalfa which are rich in mineral content.

In theory, a chemically defined diet composed of pure sources of amino acids, fatty acids, carbohydrates, vitamins and mineral salts approaches the ideal because all components are known and under complete control. Shaw (10) prepared a cariogenic diet of this type, by substituting the casein component of a purified diet by its constituent amino acids; the casein diet produced more smooth surface caries than the amino acid diet. Possibly this was because the oral clearance and rate of absorption from the intestine were more rapid for the amino acids than for the casein. Furthermore, a single amino acid such as glycine [2] exerts a significant caries-inhibiting action when added to the diet of rats, whereas glutamic acid increases tooth breakdown (11). It is advisable to use nutritionally adequate purified diets in dental research, especially in studies related to trace minerals, certainly until physiological and other nutritional problems relating to chemically defined diets (12–14) have been worked out.

This report is concerned with a study of a nutritionally adequate purified diet mixture designed for dental caries research. It may be especially useful for more precise evaluations of agents which influence the initiation and development of caries in rats. The results of a comparison of this diet (MIT-200) with a traditional caries-promoting diet (MIT-10)

Received for publication March 18, 1968.
[1] Supported by PHS Grant no. DE01438, National Institute of Dental Research. Publication no. 1122, Dept. of Nutr. and Food Sci., M.I.T.
[2] Harris, R. S., A. E. Nizel, J. M. Navia and S. K. Das 1967 Effects of dietary supplements of glycine on dental caries development in rats. 45th General Meeting, International Association for Dental Research, Chicago, Ill., p. 151 (abstract 451).

are reported in terms of body weight gain, development of various types of caries lesion, and response to 0.5% sodium trimetaphosphate (TMP), a caries-reducing agent added as supplement. Three factors were investigated: A, effect of diet MIT-200 fed postnatally; B, effect of diet MIT-200 fed prenatally; and C, effect of TMP supplement in the diet fed postnatally.

EXPERIMENTAL PROCEDURE

The sucrose, fat and lactalbumin in MIT-200 (table 1) are quite constant in composition and low in ash content. This diet also contains a special modified salt mixture based on that of Bernhart and Tomarelli (15). It provides adequate and balanced amounts of all the minerals known to be required by the rat, does not accelerate the oxidative rancidity of other dietary components, and permits the development of experimental caries.

Diet MIT-10 is composed mostly of natural foodstuffs (table 1) and has been used by us as a caries-producing diet for many years. It was used as a basis for comparison of the types and extent of caries lesions produced by the new diet. Diets MIT-200P and MIT-10P contain 0.5% sodium trimetaphosphate (TMP), a cyclic phosphate which is highly effective in reducing dental caries development in rats (16). The amount of TMP added (table 1) increased the phosphorus content of each diet by approximately 40%, to permit a comparison of the effects of this caries-reducing agent in these two types of diet.

Sixteen rats,[3] 5 days pregnant, were assigned to two groups of eight each, in a factorial design (table 2). During the remainder of the prenatal period, groups 1 to 4 were offered the natural diet, MIT-10; and groups 5 to 8 were given the purified diet, MIT-200. Diets and distilled water were offered ad libitum.

The pregnant rats were caged in groups of three in plastic cages supplied with cellulose bedding (shredded paper) until 2 days before delivery; then each was placed in a separate cage. Two days after birth, the pups in groups 1 to 4 were interchanged; those in groups 5 to 8 were interchanged; and the litters were reduced to nine pups each. Since each adopted litter contained male and female pups derived from eight different mothers, genetic differences between groups were randomized. The litters were weighed, and the foster

[3] COBS, from Charles River Laboratories, Inc., Wilmington, Mass.

TABLE 1

Composition of MIT experimental diets

Component	Purified diet no.		Natural diet no.	
	200	200P	10	10P
	%	%	%	%
Sucrose (6×, pulverized)	67.0	67.0		
Lactalbumin	20.0	20.0		
Salt mixture (MIT) [1]	3.0	3.0		
Vitamin mixture [2]	1.0	1.0		
Cottonseed oil	3.0	3.0		
Cellulose	6.0	5.5		
Whole corn meal (NE,[3] 30 mesh)			43.0	43.0
Milk powder (NE, non-fat)			20.0	20.0
Dairy plastic cream (NE)			10.0	10.0
Corn starch			4.0	3.5
Sucrose			14.0	14.0
Alfalfa meal			6.0	6.0
Liver concentrate (1:20)			2.0	2.0
Sodium chloride			1.0	0.9
Sodium trimetaphosphate (TMP)	0.0	0.5	0.0	0.5

[1] MIT salt mix: (grams per kilogram mix) $CaHPO_4$, 491.06; KCl, 141.86; NaCl, 232.85; K_2SO_4, 61.83; $MgSO_4$, 19.82; $MgCO_3$, 27.28; Fe citrate, 18.00; $MnCO_3$, 3.63; $CuCO_3$, 0.529; $ZnCO_3$, 2.70; KIO_3, 0.0506; $AlCl_3 \cdot 6H_2O$, 0.2950; and $CoCl_2 \cdot 6H_2O$, 0.0940.

[2] Vitamin mix: (grams/kilogram diet) thiamine, 0.008; riboflavin, 0.006; pyridoxine, 0.003; vitamin B_{12} (0.1%), 0.03; niacin, 0.02; Ca pantothenate, 0.03; *p*-aminobenzoic acid, 0.20; inositol, 2.00; biotin, 0.0001; menadione, 0.003; folic acid, 0.002; α-tocopherol, 0.5; ascorbic acid, 0.100; vitamin A acetate and vitamin D_2 (Roche, 1:10), 0.04; choline·HCl, 3.00; and cellulose, 4.458.

[3] NE = produced in New England.

mothers were transferred to postnatal diets (table 2).

At the time of eruption of the mandibular first molars (17 days old), the pups were weaned by removing the mothers but leaving each litter together. Each pup was weighed weekly thereafter. At 30 days of age the young rats were placed four to a cage in raised stainless steel wire-mesh cages, where they remained until the end of the experiment at 65 days of age.

These manipulations, such as early weaning (17) and caging together (18, 19) were made to increase the severity of caries and to reduce variations in caries scores. At 65 days of age, the rats were killed by guillotine; the heads were autoclaved at 121° for 15 minutes and cleaned of soft tissues. The jaws were then immersed in a solution of murexide (ammonium purpurate, 0.024% in 70% alcohol) for 16 hours, and all types of caries lesion (smooth surface, buccal-lingual, sulcal and proximal) were scored by the method of Keyes (20, 21). Statistical analysis of total caries scores was carried out using the analysis of variance methods (22).

RESULTS AND DISCUSSION

Body weights. To follow the growth of rats in the different experimental groups closely, the animals were weighed every 3 days during the first 30 days of life and weekly thereafter (table 3). At 2 days of age, the weights of the pups whose mothers had been fed the purified diet (groups 5 to 8) were significantly higher than of the pups whose mothers had received the natural diet. This effect was lost by the time the pups had reached 30 days of age, how-

TABLE 2

Experimental design showing distribution of litters into experimental groups

Group	No. of rats	Treatment [1]	Prenatal (— 15 to 2 days) MIT diet no.	Postnatal (2 to 65 days) MIT diet no.
1	18	Control	10 [2]	10
2	18	A	10	200
3	18	C	10	10 P
4	18	AC	10	200 P
5	18	B	200	10
6	18	AB	200	200
7	18	BC	200	10 P
8	18	ABC	200	200 P

[1] A = diet MIT-200 fed postnatally (2 to 65 days of age); B = diet MIT-200 fed prenatally (— 15 to 2 days of age); C = 0.5% TMP supplement postnatally (2 to 65 days of age).
[2] See table 1 for diet compositions and supplements.

TABLE 3

Mean body weights of rats in experimental groups

Group	Treatment	Litters randomized [1]	Weaning [2]	Transferred to raised bottom cages [3]	65 days of age [4]		No. of rats killed
					Males	Females	
		g	*g*	*g*	*g*	*g*	
1	Control	7.2	31.4	71.8	233 ± 28 [5]	186 ± 14	15
2	A	7.1	34.1	78.1	263 ± 33	193 ± 19	18
3	C	6.9	25.4	63.0	218 ± 31	167 ± 19	15
4	AC	7.2	37.1	78.6	265 ± 37	201 ± 18	16
5	B	7.6	31.6	72.3	231 ± 33	164 ± 36	18
6	AB	7.8	34.8	84.2	270 ± 19	195 ± 39	18
7	BC	7.5	31.3	69.1	215 ± 29	166 ± 18	18
8	ABC	8.0	33.8	80.1	262 ± 27	199 ± 28	18

[1] Age 2 days, males and females.
[2] Age 17 days, males and females.
[3] Age 30 days, males and females.
[4] All rats killed.
[5] Mean ± SD.

ever. At all the age intervals, the animals (groups 2 and 6) fed the purified diet postnatally were heavier than the corresponding animals (groups 1 and 5) fed the natural diet postnatally. The heaviest group, 6, was that which had been maintained with the purified diet during both the prenatal and postnatal periods. The lowest weights were noted in groups 3 and 7 fed the phosphate-supplemented natural diet. The body weight gains of both male and female rats in the different groups followed the same patterns postnatally as prenatally. The rats fed the purified diet had higher body weight gains than those offered the natural diet. Intragroup variations in body weight were so large that intergroup differences could not be established statistically. Both diets were readily accepted by the rats, and no important differences in food consumption by the different groups were noted.

Caries score evaluation. The average caries scores for all surfaces (buccal-lingual, sulcal and proximal) are tabulated in tables 4 and 5. In table 5 are listed also the average accumulated scores for all lesions grouped according to depth of penetration of the lesions. When diet MIT-200 was offered both pre- and postnatally (group 6), the enamel scores were significantly higher than when diet MIT-10 was offered under the same conditions (group 1), although no significant difference was obtained using the dentinal scores.

The slight dentinal lesions (D_s) as well as the medium dentinal lesions (D_m) are useful for evaluating caries-producing diets. Figures 1 and 2 present graphically the total D_s and D_m lesions, respectively, as well as the proportions contributed by the buccal-lingual, sulcal and proximal surfaces. The total caries score used combines

TABLE 4

Average caries score for all surfaces of rat molars

Group	Treatment	Buccal-lingual				Sulcal				Proximal			
		E [1]	D_s[2]	D_m[3]	D_x[4]	E	D_s	D_m	D_x	E	D_s	D_m	D_x
1	Control	14.3	7.6	5.7	3.5	30.6	23.3	12.8	5.4	4.3	1.5	0.5	0.1
2	A	14.6	4.5	1.7	0.3	32.3	25.5	12.0	0.8	4.2	1.7	0.3	0.0
3	C	13.2	3.2	2.0	1.2	28.7	18.7	6.5	1.7	5.8	2.3	0.0	0.0
4	AC	17.0	0.4	0.0	0.0	31.6	22.9	5.4	0.0	4.7	1.2	0.2	0.0
5	B	10.1	3.9	2.1	1.2	29.0	20.4	11.4	3.9	5.0	1.9	0.2	0.0
6	AB	17.0	6.5	2.7	0.8	37.1	29.1	14.9	1.5	5.0	2.3	0.5	0.0
7	BC	13.0	2.3	0.7	0.1	29.5	20.9	6.1	0.9	6.5	2.6	0.2	0.0
8	ABC	11.9	0.2	0.0	0.0	31.9	22.8	7.6	0.0	5.1	0.6	0.1	0.0

[1] E = enamel lesion.
[2] D_s = slight dentinal involvement.
[3] D_m = lesion extending into dentin.
[4] D_x = extensive dentinal involvement.

TABLE 5

Combined average caries score for all lesions (buccal-lingual + sulcal + proximal) based on depth of penetration

Group	Treatment	Cumulative scores (all lesions)		
		E [1]	D_s[2]	D_m[3]
1	Control	49.2 ± 3.6 [4]	32.4 ± 3.1	19.0 ± 2.6
2	A	51.1 ± 3.1	31.7 ± 2.6	14.0 ± 1.9
3	C	47.7 ± 3.1	24.2 ± 3.1	8.5 ± 1.9
4	AC	53.3 ± 2.3	24.5 ± 1.6	5.6 ± 1.2
5	B	44.1 ± 3.7	26.2 ± 2.8	13.7 ± 1.8
6	AB	59.1 ± 2.9	37.9 ± 2.1	18.1 ± 2.7
7	BC	49.0 ± 3.1	25.8 ± 2.2	7.0 ± 1.5
8	ABC	48.9 ± 2.1	23.6 ± 1.8	7.7 ± 1.6

[1] E = enamel lesions.
[2] D_s = slight dentinal involvement.
[3] D_m = lesion extending into dentin.
[4] Values are scores ± SE.

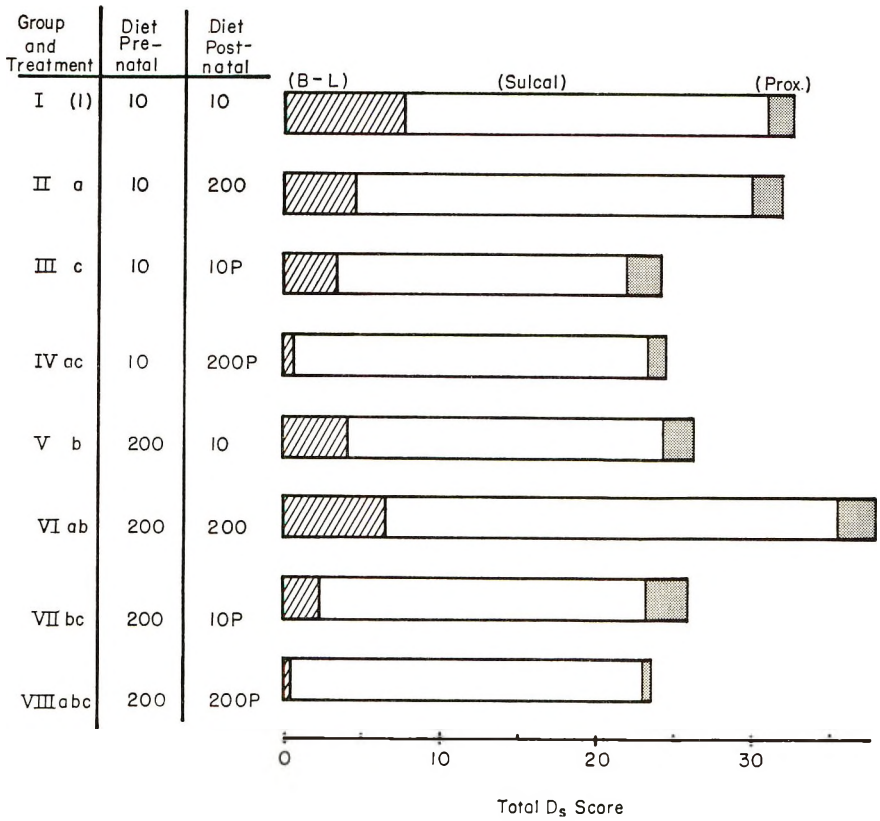


Fig. 1 Accumulated D_s (slight dentinal) average caries lesion from buccal-lingual, sulcal and proximal rat molar surfaces.

all the carious enamel or dentinal areas of buccal, lingual, sulcal and proximal molar surfaces. It is a measure of the overall caries attack on the dentition (23, 24).

Statistical analysis of these data (table 6) indicated that the cariostatic effect of the trimetaphosphate was highly significant regardless of whether it was tested with the natural or the purified diet. The only other data that were significant related to treatment ABC (group 8); this may be explained as the effect on dentinal caries which resulted from feeding the purified sugar prenatally. The effect of feeding the purified diet prenatally was to decrease the caries in the rats fed the natural diet postnatally (group 1 versus group 5) and to increase caries in the rats continued on the same diet postnatally (group 2 versus group 6). This difference in response possibly accounts for the lack of significance of factor B in the analysis of variance and for the significance of the three-factor interaction. Trimetaphosphate also contributed to this interaction, since its cariostatic effect was more pronounced, when tested with the purified diet (group 6 versus group 8), than with the natural diet (group 1 versus group 3).

The cariostatic effect of trimetaphosphate was detected in all types of lesions (fig. 1); it was most dramatic in controlling the smooth surface lesions on the buccal and lingual surfaces, and less effective in controlling proximal lesions. This effect of trimetaphosphate was clearly seen in deeper D_m and D_x lesions (tables 4 and 7) where the scores of those groups given this treatment (groups 3, 4, 7 and 8) were significantly lower than those not receiving this treatment (groups 1, 2, 5 and 6). When the deeper lesions (D_m) are consid-

ered, the caries-promoting properties of the purified diet (MIT-200) decreased in comparison with the natural diet (MIT-10), and the trimetaphosphate was able to reduce caries equally well when fed in either diet. Thus, the significance of the three-factor interaction disappears, and only the interaction AB is significant. This was due

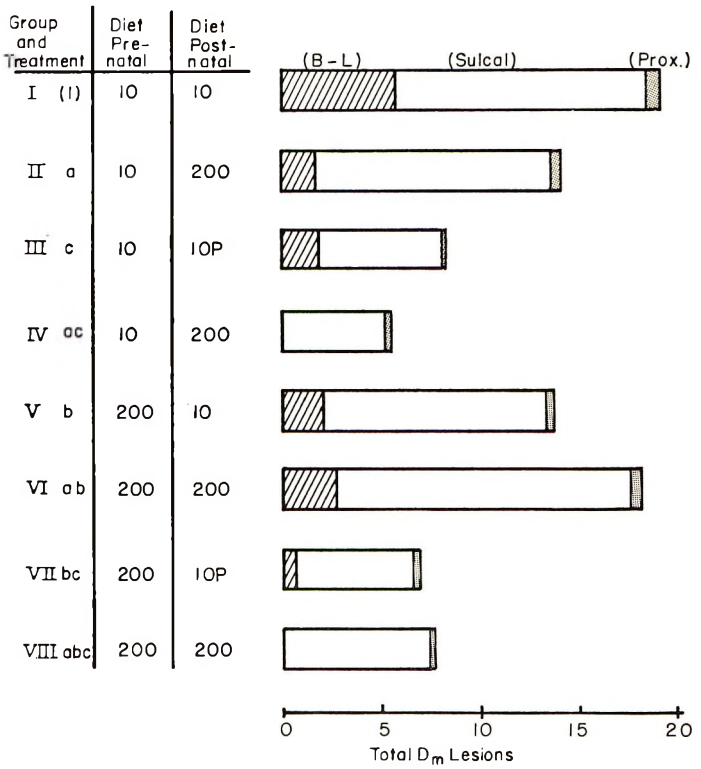


Fig. 2 Accumulated D_M (moderate dentinal) average caries lesions from buccal-lingual, sulcal and proximal rat molar surfaces.

TABLE 6

Analysis of variance of total D_s[1] scores for rat molars

Source of variation	Sum of squares	df [2]	Mean square	F
All treatments	2750.0	7	392.9	4.24 **
A	154.1	1	154.1	1.66 ns [3]
B	1.2	1	1.2	1.30 ns
C	1687.5	1	1687.5	18.2 **
AB	182.5	1	182.5	1.97 ns
AC	313.6	1	313.6	3.38 ns
BC	0.3	1	0.3	0.01 ns
ABC	410.7	1	410.7	4.44 *
Residual	10,369.2	112	92.6	
Total	13,119.2	119		

[1] D_s = slight dentinal involvement.
[2] df = degrees of freedom.
[3] ns = not significant.
* $P \leq 0.05$.
** $P \leq 0.001$.

TABLE 7

Statistical evaluation of individual comparisons between accumulated caries scores from experimental groups using the least significant difference (25) [1]

	Total enamel lesions							
Treatment	AB	AC	A	Control	BC	ABC	C	B
Score	59.2	53.3	51.1	49.2	49.0	48.9	47.7	44.1

	Total D_s[2] lesions							
Treatment	AB	Control	A	B	BC	AC	C	ABC
Score	37.9	32.4	31.7	26.3	25.8	24.5	24.3	23.6

	Total D_m[3] lesions							
Treatment	Control	AB	A	B	C	ABC	BC	AC
Score	19.9	18.1	14.0	13.7	8.5	7.7	7.0	5.6

[1] The underlines joining the groups denote that these treatments have not been shown to be significant ($P \leq 0.05$). Any two means not underscored by the same line are significantly different.
[2] D_s = slight dentinal involvement.
[3] D_m = lesion extending into dentin.

to the effect of the purified diet, fed prenatally, upon the caries experience of rats fed the natural diet postnatally.

No explanation is being offered at this time for the effect of the purified diet fed prenatally. Larson and Zipkin (19) have suggested that the environment during the first hours of life may affect the later development of caries. Possibly the purified diet had a selective influence on the cariogenic oral flora in the mother rats, or possibly diets MIT-10 and MIT-200 may have influenced the caries experience of the offspring to different degrees.

The distribution and depth of penetration of the carious lesions were quite different between the two diets fed. Rats fed the purified diet had higher scores in the buccal-lingual and sulcal surfaces, but the degree of penetration of the lesions into the deeper portions of the dentin was drastically less in rats fed this diet than in rats fed natural diets.

Sodium trimetaphosphate was fed at a level of 0.5%, which is less than one-half the amount added in previous studies (16); yet it was effective in reducing caries. It was equally effective as a supplement to the purified diet (MIT-200) as to the natural diet (MIT-10). This was better demonstrated by its capacity to reduce the depth of penetration of the lesion, than by its ability to prevent the carious attack. We now consider the effect of trimetaphosphate when fed to rats to be exerted mostly by a local action on the tooth surface and related to a remineralization process taking place at the enamel surface which could influence the colonization of the microflora responsible for caries. In this respect, trimetaphosphate is different from other phosphate compounds in terms of mechanism. Research currently under way should contribute to the understanding of the cariostatic effect of trimetaphosphate. The purified diet (MIT-200) used in these stud-

ies has been shown to be both nutritionally adequate and capable of promoting all types of carious lesions in rats.

LITERATURE CITED

1. Shaw, J. H. 1947 Carious lesions in cotton rat molars. I. The role of mechanical factors studied by the extraction of antagonistic molars. J. Dent. Res., 26: 47.
2. Haldi, J., W. Wynn, J. Shaw and R. F. Sognnaes 1958 Influence of salt mixture added to cariogenic diets of comparable sucrose content. J. Nutr., 66: 333.
3. Constant, M. A., H. W. Sievert, P. H. Phillips and C. A. Elvehjem 1954 Dental caries in the cotton rat. IV. The effect of tooth maturity and minerals on caries production by semisynthetic diets. J. Nutr., 53: 29.
4. Stephan, R. M., and M. R. Harris 1955 Location of experimental caries on different tooth surfaces in the Norway rat. In: Advances in Experimental Caries Research Symposium, Boston, 1953. American Association for the Advancement of Science, Washington, D. C.
5. Ludwig, T. G., R. S. Malthus and W. B. Healy 1962 An association between soil conditions and dental caries in rats. Nature, *194:* 456.
6. Van Reen, R., C. A. Ostrom and V. J. Berzinskas 1962 The cariostatic effect of dietary phosphate in the presence or absence of fluoride in the drinking water. Arch. Oral Biol., 7: 587.
7. Gustafson, G., E. Stelling and E. Brunius 1963 The cariogenetic effect of variations in salt mixture in a synthetic cariogenic diet. Acta Odontol. Scand., *21:* 297.
8. Larson, R. H. 1963 A semipurified caries-test diet for rats. J. Dent. Res., 42: 178.
9. König, K. G., and H. R. Mühlemann 1964 The effects of some dietary mineral supplements on experimental rat caries. Helv. Odontol. Acta, *I:* (suppl.) 41.
10. Shaw, J. 1967 Influence of casein replacement by amino acid mixture on experimental dental caries in rats and on the periodontal syndrome in rice rats. J. Dent. Res., 45: 1810.
11. Nordstrom, J. W., and E. L. R. Stokstad 1967 Destruction of molar teeth in rats fed amino acid diets. J. Nutr., *93:* 189.
12. Salmon, W. D. 1964 Relative potency of diets containing amino acids and proteins for promoting growth of rats. J. Nutr., 82: 76.
13. Rogers, Q. R., and A. E. Harper 1965 Amino acid diets and maximal growth in the rat. J. Nutr., 87: 267.
14. Ranhotra, G. S., and B. C. Johnson 1965 Effect of feeding different amino acid diets on growth rate and nitrogen retention of weanling rats. Proc. Soc. Exp. Biol. Med., *118:* 1197.
15. Bernhart, F. W., and R. M. Tomarelli 1966 A salt mixture supplying the National Research Council estimates of the mineral requirement of the rat. J. Nutr., 89: 495.
16. Harris, R. S., A. E. Nizel and N. B. Walsh 1967 The effect of phosphate structure on dental caries development in rats. J. Dent. Res., *46:* 290.
17. Madsen, K. O., and E. J. Edmons 1963 Use of twelve-day-old cotton rats for dental caries studies. J. Dent. Res., *42:* 867.
18. Larson, R. H. 1964 Caries development in rats of different ages with controlled flora. Arch. Oral Biol., *9:* 705.
19. Larson, R. H., and I. Zipkin 1965 Influence of early environment of newborn rats on development of dental caries. Arch. Oral Biol., *10:* 23.
20. Keyes, P. 1958 Dental caries in molar teeth of rats. I. J. Dent. Res., 37: 1077.
21. Keyes, P. 1958 Dental caries in molar teeth of rats. II. J. Dent. Res., 37: 1088.
22. Dixon, W. J., and F. J. Massey 1957 Introduction to Statistical Analysis, ed. 2. McGraw-Hill Book Company, New York.
23. Chung, C. S., and R. H. Larson 1967 Factors and inheritance of dental caries in the rat. J. Dent. Res., *46:* 559.
24. Chung, C. S., R. H. Larson and B. J. Gross 1968 Perinatal and growth-associated factors influencing dental caries in rats. J. Dent. Res., *47:* 139.
25. Duncan, D. B. 1955 Multiple range and multiple *F* tests. Biometrics, *11:* 1.

Effect of Diet and Manganese Level on Growth, Perosis and ^{54}Mn Uptake in Chicks [1]

E. A. SETTLE,[2] F. R. MRAZ, C. R. DOUGLAS [3] AND J. K. BLETNER
Poultry Department, University of Tennessee, Knoxville, Tennessee, and Agricultural Research Laboratory of the University of Tennessee, Oak Ridge, Tennessee [4]

ABSTRACT Broiler-type chicks were used to study the effect of graded levels of manganese in a purified diet and in two practical-type diets (one without and one with 14.5% feather meal) on growth, incidence of perosis, the in vivo uptake of ^{54}Mn by certain tissues and in vitro uptake of ^{54}Mn by erythrocytes. Generally, growth was unaffected by the manganese levels except that chicks fed the purified diet containing no added manganese achieved very poor growth. The incidence of perosis was decreased by manganese additions but was not eliminated. An inverse relationship was observed between dietary manganese and the percentage of ^{54}Mn taken up by liver, kidney, spleen and muscle. The first added increment of manganese in the diet had the greatest effect in reducing the uptake of ^{54}Mn by intramuscular dose. The inconsistent ^{54}Mn tibia uptake was probably affected by bone size. Erythrocytes incubated with ^{54}Mn took up some of the isotope, but the amount failed to reflect the manganese content of the diet. No appreciable amount of manganese appeared to be tied up by the feather meal diets as evidenced by tissue and excretion data.

Diets containing 14.5% hydrolyzed feather meal have failed to produce normal growth (1) and frequently have produced a high incidence of perosis in battery-reared chicks.[5] Since the diets used were well fortified with manganese and other perosis-preventing factors it was considered possible that feather meal could contain some factor or factors which made the manganese unavailable or which inhibited the utilization of dietary manganese. Furthermore, the possibility of detecting a manganese deficiency in vitro was suggested by the response of selenium-deficient ovine erythrocytes to selenium in vitro (2), and the demonstration that duck erythrocytes were capable of taking up ^{54}Mn (3).

Experiments were therefore initiated to study growth, incidence of perosis, ^{54}Mn uptake of erythrocytes in vitro and tissue absorption and deposition of ^{54}Mn in chicks fed various types of diets containing graded levels of manganese.

EXPERIMENTAL

One-day-old broiler-type chicks housed in electrically heated, raised wire-floored battery brooders, were supplied feed and water ad libitum. The birds were weighed and observed for perosis at weekly intervals for 4 weeks. All supplemental manganese was feed grade $MnSO_4$ and all radioactive manganese was carrier-free $^{54}MnCl_2$.

The composition and calculated analyses of the feather meal and soybean oil meal basal diets are given in table 1, and the composition of the purified basal diet in table 2. The calculated manganese content was 0.0, 7.9, and 14.5 mg/kg for the purified, feather meal and soybean oil meal basal diets, respectively. In experiment 1 the feather meal and soybean oil meal diets were fed alone, and with manganese added at the expense of the entire diet, in amounts calculated to bring the total manganese content to about 28, 55, 110, 220 and 330 mg/kg to provide the 12 treatments. A randomized split block experimental design was used with four replications per treatment. Six male and six

Received for publication May 29, 1968.

[1] Published with the approval of the Dean of the Agricultural Experiment Station, Institute of Agriculture of the University of Tennessee.

[2] Present address: 2525 S. Sheridan #22, Denver, Colorado 80227.

[3] Present address: Agricultural Extension Service, Poultry Department, University of Tennessee, Knoxville, Tennessee.

[4] Agricultural Research Laboratory which is operated by Tennessee Agricultural Experiment Station for the U.S. Atomic Energy Commission under Contract no. AT-40-1-GEN-242.

[5] Settle, E. A., J. F. Stephens and J. K. Bletner, unpublished data. Tennessee Agricultural Experiment Station.

TABLE 1

Composition and calculated analyses of the feather meal and soybean oil meal basal diets

Ingredients	Feather meal basal	Soybean oil meal basal
	%	%
Yellow corn	68.265	58.440
Alfalfa meal (17% protein)	2.500	2.500
Fish meal (65% protein)	5.700	5.700
Feather meal (85% protein)	14.500	—
Soybean oil meal (50% protein)	—	28.210
Fat, stabilized, fancy bleachable	—	1.800
Defluorinated rock phosphate	1.200	1.200
Limestone	1.000	1.000
Salt	0.500	0.500
Vitamin mix [1,2]	4.000	0.600
DL-Methionine	0.065	0.050
L-Lysine supplement (220 g/kg)	2.270	—
Total	100.000	100.000
Calculated analyses [3]		
Productive energy, kcal/kg	2123	2130
Crude protein, %	23.71	23.66
Calcium, %	1.14	1.17
Total phosphorus, %	0.68	0.72
Inorganic phosphorus, %	0.55	0.54
Manganese, mg/kg	7.81	14.48

[1] Supplied the following per kilogram of feather meal diet: vitamin D_3, 792 ICU; vitamin A, 4,400 IU; riboflavin, 8.8 mg; vitamin B_{12}, 11.33 μg; choline chloride, 770 mg; niacin, 88 mg; Ca D-pantothenate, 8.8 mg; aureomycin, 12.1 mg; and arsanilic acid, 83.6 mg.

[2] Supplied the following per kilogram of soybean oil meal diet: vitamin D_3, 748 ICU; vitamin A, 4,162 IU; riboflavin, 4.73 mg; vitamin B_{12}, 11 μg; choline chloride, 468 mg; niacin, 40.2 mg; Ca D-pantothenate, 7 mg; aureomycin, 11.88 mg; and arsanilic acid, 83.3 mg.

[3] Calculations based on average analysis of feedstuffs according to Hubbell (10) except for manganese which were based on data from Heuser (11) and unpublished data in this laboratory.

TABLE 2

Composition of purified basal diet

Ingredients	
	%
Glucose	59.88
Isolated soybean protein	27.00
Corn oil	3.00
Cellulose	3.00
Vitamin mix [1]	1.22
Mineral mix [2]	5.80
Glycine	0.30
Methionine hydroxy analogue	0.70
Total	100.90

[1] Supplied the following per kilogram of diet: inositol, 250.0 mg; niacin, 50.0 mg; calcium pantothenate, 20.0 mg; pyridoxine·HCl, 4.5 mg; folic acid, 4.0 mg; menadione sodium bisulfite, 1.52 mg; biotin, 0.20 mg; vitamin B_{12}, 54 μg diluted to 16.0 g; α-tocopheryl acetate, 66.0 mg; vitamin A, 5400 IU; vitamin D_3, 980 ICU; thiamine·HCl, 11.0 mg; riboflavin, 11.0 mg; choline chloride, 1540.0 mg; and santoquin, 0.25 g.

[2] Supplied the following per kilogram of diet: (in grams) $CaHPO_4$, 17.0; $CaCO_3$, 18.3; KH_2PO_4, 13.8; NaCl, 6.0; and $MgSO_4$, 2.5; (in milligrams) $FeSO_4 \cdot 7H_2O$, 333.0; KI, 2.6; $CuSO_4 \cdot 5H_2O$, 16.7; $CoCl_2 \cdot 6H_2O$, 1.7; $Na_2MoO_4 \cdot 2H_2O$, 8.3; and ZnO, 74.7.

female chicks were randomly assigned to each pen. The purified diet, without and with 55.0 mg added manganese per kilogram, was used to study the ability of the radioisotope technique to detect manganese deficiency. Each purified diet was fed to one pen of six male and six female chicks.

The uptake in vitro of ^{54}Mn by erythrocytes was tested by using the method of Wright and Bell (2). Blood samples from three male and three female nonperotic chicks per treatment and from a like sample of perotic birds, when available, were obtained when the chicks were 28 or 29 days old.

During week 5, groups of five representative cockerels, fed either the feather meal or soybean oil meal diets containing no added manganese and 110 mg manganese/kg, or the two purified diets, were orally dosed with 25 μCi of ^{54}Mn (as $^{54}MnCl_2$). The excreta samples were collected daily for 96 hours at which time the birds were killed. Samples of liver, kidney, spleen, blood and tibia were collected and their ^{54}Mn contents determined. The excreta samples were dried; they were then ashed, dissolved in concentrated hydrochloric acid and aliquot samples were

taken to determine ^{54}Mn content. The entire tibia was dried, ashed and counted.

Experiment 2 was a continuation and extension of experiment 1 using the three basal diets. These diets were fed alone, and in addition manganese was added to each diet at the expense of the entire diet in amounts calculated to bring the total manganese content to about 55, 110, 220 and 440 mg manganese/kg of feed. A randomized block experimental design was used with three replications per treatment using eight male and seven female chicks randomly assigned to each pen. The composition of the basal diets was the same as in experiment 1.

A severity index for the incidence of perosis was set up using the following code: 1 (slightly slipped tendon); 2 (slipped tendon); and 3 (slipped tendon and twisted leg).

Blood samples for the in vitro study were collected as in experiment 1. Five representative cockerels from each treatment were dosed intramuscularly with 10 μCi of ^{54}Mn. Excreta samples were collected for 48 hours at which time the birds were killed. Samples of liver, kidney, spleen, muscle, blood and tibia were taken. The samples were processed as described in experiment 1 except that aliquot samples of tibia were used for counting. The ^{54}Mn content of all tissue samples was determined in a well-type scintillation counter equipped with a 7.6 cm by 7.6 cm sodium iodide (Th) crystal.

Data obtained from these experiments were analyzed according to an analysis of variance procedure suggested by Snedecor (4). Significant differences were determined by the use of Duncan's multiple range test (5).

RESULTS AND DISCUSSION

The average 4-week body weights and the incidence of perosis in experiments 1 and 2 are given in tables 3 and 4, respectively. In experiment 1 the average weight of chicks fed the soybean oil meal diets containing 110 and 330 mg manganese/kg did not differ significantly ($P > 0.05$) from that of chicks fed the feather meal diets containing 55 mg manganese/kg. In experiment 2 the average weights of all chicks fed the soybean oil meal diets were significantly larger than those of chicks fed the feather meal or the purified diets. Chicks fed the feather meal diets had about the same average body weights in both experiments; however, those fed the soybean oil meal diets were heavier in experiment 2 than in experiment 1. Thus, the difference in body weight between chicks fed the feather meal diets and chicks fed the soybean oil meal diets was greater in experiment 2 than in experiment 1. In experiment 2, the average body weight of chicks fed the purified diet containing 55, 110, 220 and 440 mg manganese/kg did not differ significantly ($P > 0.05$) from those of chicks fed the feather meal diets.

Increasing the level of manganese in the feather meal and soybean oil meal diets had no consistent effect on average 4-week body weights. The first addition of manganese (55 mg/kg) to the purified diet resulted in a significant ($P < 0.05$) increase in growth, but further additions of manganese failed to produce additional growth responses.

TABLE 3

Growth and incidence of perosis (exp. 1)

Diet and calculated manganese	Avg [1] 4-week body wt [2]	Chicks with perosis
mg/kg	*g*	*%*
Feather meal		
7.9	464 [e]	54
27.5	468 [e]	29
55.0	492 [cd]	12
110.0	482 [de]	27
220.0	483 [de]	31
330.0	484 [de]	15
Soybean oil meal		
14.5	536 [a]	29
27.5	536 [a]	27
55.0	528 [ab]	12
110.0	516 [abc]	29
220.0	532 [a]	25
330.0	509 [bc]	21
Purified		
0.0	349 [x]	100
55.0	552 [y]	8

[1] Averages for the feather meal and soybean oil meal diets are of four replicate pens each containing six male and six female broiler-type chicks. Averages for the purified diets are based on one pen of six male and six female broiler-type chicks.

[2] Means bearing unlike superscripts differ significantly ($P < 0.05$). Data from the purified diets are not compared with those from the feather meal and soybean oil meal diets because the experiment with the purified diet was conducted at a later date.

TABLE 4

Growth and incidence of perosis (exp. 2)

Diet and calculated manganese	Avg [1] 4-week body wt [3]	Perosis			Chicks with perosis
		Severity index [2]			
		1	2	3	
mg/kg	*g*	*no. of chicks*			*%*
Feather meal					
7.9	488 [b]	2	6	6	31
55.0	486 [b]	4	1	2	16
110.0	505 [b]	4	2	0	13
220.0	504 [b]	5	2	1	18
440.0	486 [b]	7	1	2	22
Soybean oil meal					
14.5	568 [a]	5	4	1	22
55.0	562 [a]	2	1	1	9
110.0	582 [a]	4	2	2	18
220.0	567 [a]	4	2	3	20
440.0	563 [a]	5	1	2	18
Purified					
0.0	341 [c]	3	7	35	100
55.0	507 [b]	3	2	0	11
110.0	507 [b]	3	0	1	9
220.0	489 [b]	3	2	2	13
440.0	502 [b]	3	4	0	16

[1] Averages are of three replicate pens each containing eight male and seven female broiler-type chicks.
[2] Code for severity index: 1 (slightly slipped tendon); 2 (slipped tendon); and 3 (slipped tendon and twisted leg).
[3] Means bearing unlike superscripts differ significantly ($P < 0.05$).

The chicks fed the unsupplemented feather meal diet had a high incidence of perosis. Adding manganese to this diet reduced, but did not eliminate, the incidence of perosis. The lowest incidence of perosis in chicks fed the feather meal diets was observed when the diet contained 55 mg manganese/kg in experiment 1 and 110 mg manganese/kg in experiment 2.

The incidence of perosis among chicks fed the soybean oil meal diets was higher than expected with these diets. In both experiments the incidence of perosis was lowest when the diet contained 55 mg manganese/kg, the recommended level of manganese (6) for chick diets.

The addition of 55 mg manganese/kg of purified diet markedly reduced the incidence of perosis but additional manganese failed to completely prevent the condition. The incidence of perosis tended to be lower among chicks fed the purified diets containing 55 or more milligrams manganese per kilogram than it was among chicks fed the feather meal or soybean oil meal diets.

Although the data obtained from orally (table 5) and intramuscularly (table 6) administered ^{54}Mn differed in magnitude of the percentage uptake of ^{54}Mn, the trend of results was in agreement with the exception of the tibia data of the birds fed the feather meal and soybean oil meal diets. The addition of manganese to the diet significantly reduced ($P < 0.05$) the uptake of orally administered ^{54}Mn by the tissues with the exception of the tibiae of birds fed the feather meal and purified diets (table 5). In both experiments the addition of manganese to the purified diet increased ^{54}Mn uptake by tibiae, which might be related to the poor growth experienced on the diet without added manganese. Chicks fed the manganese-supplemented purified diets were much larger, and therefore, had more bone to take up ^{54}Mn.

In experiment 2, the tibia values of chicks fed the feather meal and purified diets, expressed as percentage dose per gram of tibia, ranged from 0.250 to 0.301 and were not related to the manganese level of the diet. Based on a percentage per tibia basis (table 6), however, the addition of manganese to both basal diets increased total tibia uptake significantly

TABLE 5

Percentage of orally administered ^{54}Mn dosage recovered in certain tissues and excreta (exp. 1)

Diet and calculated manganese	Tibia	Liver	Kidney	Spleen	96-hr excretion
mg/kg	*10^{-2}% /tibia* [1]	*10^{-2}% /g* [1]	*10^{-2}% /g* [1]	*10^{-2}% /g* [1]	%
Feather meal					
7.9	1.46 [a]	0.97 [a]	1.28 [a]	0.28 [a]	90
110.0	1.16 [a]	0.20 [b]	0.28 [b]	0.05 [b]	100
Soybean oil meal					
14.5	3.12 [a]	0.98 [a]	0.98 [a]	0.23 [a]	99
110.0	0.90 [b]	0.16 [b]	0.20 [b]	0.05 [b]	98
Purified					
0.0	1.72 [a]	2.30 [a]	1.87 [a]	0.40 [a]	66
55.0	3.28 [b]	0.54 [b]	0.60 [b]	0.12 [b]	88

[1] For various manganese levels within a diet type, means bearing unlike superscripts are significantly different ($P < 0.05$).

TABLE 6

Percentage of intramuscularly administered ^{54}Mn dosage recovered in certain tissues and excreta (exp. 2)

Diet and calculated manganese	Tibia	Liver	Kidney	Spleen	Muscle	48-hr excretion
mg/kg	*% /tibia* [1]	*% /g* [1]	*% /g* [1]	*% /g* [1]	*10^{-1}% /g* [1]	%
Feather meal						
7.9	0.96 [a]	0.45 [a]	0.61 [a]	0.20 [a]	0.32 [a]	21
55.0	1.04 [b]	0.30 [b]	0.41 [b]	0.12 [b]	0.25 [b]	39
110.0	1.06 [b]	0.26 [c]	0.38 [c]	0.09 [c]	0.22 [c]	41
220.0	1.08 [c]	0.18 [d]	0.35 [d]	0.08 [d]	0.19 [d]	48
440.0	1.01 [b]	0.16 [e]	0.33 [e]	0.07 [e]	0.18 [d]	52
Soybean oil meal						
14.5	0.94 [a]	0.48 [a]	0.60 [a]	0.18 [a]	0.31 [a]	27
55.0	0.91 [a]	0.33 [b]	0.44 [b]	0.13 [b]	0.25 [b]	37
110.0	0.90 [a]	0.20 [c]	0.34 [c]	0.10 [c]	0.18 [c]	45
220.0	0.96 [a]	0.17 [d]	0.25 [d]	0.07 [d]	0.16 [d]	49
440.0	0.87 [a]	0.12 [e]	0.22 [e]	0.05 [e]	0.13 [e]	52
Purified						
0.0	0.68 [a]	0.90 [a]	1.84 [a]	0.51 [a]	0.79 [a]	12
55.0	0.99 [c]	0.28 [b]	0.38 [bc]	0.12 [b]	0.22 [b]	32
110.0	0.98 [c]	0.24 [c]	0.36 [cd]	0.10 [c]	0.22 [b]	36
220.0	0.86 [b]	0.16 [e]	0.33 [d]	0.09 [d]	0.21 [b]	44
440.0	0.98 [c]	0.19 [d]	0.40 [b]	0.08 [e]	0.18 [c]	46

[1] For various manganese levels within a diet type, means bearing unlike superscripts are significantly different ($P < 0.05$).

($P < 0.05$). Chicks fed the soybean oil meal diet containing 14.5, 55, 110, 220 and 440 mg manganese/kg took up 0.258, 0.249, 0.228, 0.224 and 0.204% of the dose of ^{54}Mn per gram of tibia, respectively. The first and second uptake values did not differ significantly ($P > 0.05$), but they did differ significantly ($P < 0.05$) from the other values. The same was true for the third and fourth values. When considered on a per tibia basis the differences were not significant ($P > 0.05$). Parker et al. (7) fed a semipurified diet with two levels of manganese and reported that the tibiae of manganese-deficient chicks accumulated more $^{52\text{-}54}$Mn than those of normal chicks when calculated on a percentage dose per gram of tibia basis. There is no apparent explanation for the failure of the tibiae of chicks fed the feather meal and purified

diets to take up ^{54}Mn as did tibiae from chicks fed the soybean oil meal diet, or as reported by Parker et al. (7). Tibiae of birds dosed intramuscularly and fed diets containing feather meal had consistently higher ^{54}Mn uptake values than tibiae of birds fed the soybean oil meal diets. The tibiae from chicks fed the soybean oil meal diets increased in weight as the manganese level in the diet increased.

In all soft tissues, the first added increment of manganese in the diet had the greatest effect in reducing the uptake of ^{54}Mn administered intramuscularly. As the manganese level of the diet increased, the ^{54}Mn uptake by the soft tissues decreased ($P < 0.05$).

The total excretion of ^{54}Mn followed more closely the calculated values of manganese in the diet than did the percentage of ^{54}Mn of the tissues. The percentage of the dose excreted in the 48-hour postadministration period in experiment 2 was much less than in the 96-hour postadministration period of experiment 1. That the difference was due to the method of administration of the isotope (intramuscularly in experiment 2 and orally in experiment 1) rather than a time factor is suggested by the larger uptake of ^{54}Mn by the various tissues, and by a similar difference reported by Mohamed and Greenberg (8) for orally and subcutaneously administered ^{56}Mn.

Blood plasma ^{54}Mn values are not shown since no appreciable amounts of radioactivity were found in chicks after ^{54}Mn by oral or intramuscular dose. Hill (9) noted that the concentration of radioactive manganese in the blood was low when the isotope was administered orally to laying pullets. Erythrocytes incubated in vitro with ^{54}Mn took up about 3 to 8% of the isotope, but the amount taken up failed to reflect the manganese content of the diet or the presence or absence of perosis in the chick, and the data are not reported. The poor uptake of ^{54}Mn normally in vivo may have been the contributory factor to the ineffectiveness of the erythrocyte uptake technique as a means of detecting manganese deficiency.

No appreciable amount of manganese appeared to be tied up by the feather meal diets as evidenced by tissue and excretion data.

LITERATURE CITED

1. Stephens, J. F., J. K. Bletner and O. E. Goff 1961 Supplementation and processing methods of feather meal used in broiler diets. Tenn. Farm Home Sci., Prog. Rep., *39:* 2.
2. Wright, P. L., and M. C. Bell 1963 Selenium and vitamin E influence upon the in vitro uptake of Se-75 by ovine blood cells. Proc. Soc. Exp. Biol. Med., *114:* 379.
3. Norris, G. R., and J. R. Klein 1961 Incorporation of manganese into duck erythrocytes. Proc. Soc. Exp. Biol. Med., *106:* 288.
4. Snedecor, G. W. 1956 Statistical Methods, ed. 5. The Iowa State University Press, Ames.
5. Duncan, D. B. 1955 Multiple range and multiple F tests. Biometrics, *11:* 1.
6. National Research Council 1960 Nutrient requirements of poultry, publ. no. 827. National Academy of Sciences — National Research Council, Washington, D. C.
7. Parker, H. E., F. N. Andrews, C. W. Carrick, R. D. Creek and S. M. Hauge 1955 Effect of manganese on bone formation studied with radioactive isotopes. Poultry Sci., *34:* 1154.
8. Mohamed, M. S., and D. M. Greenberg 1943 A tracer study with Mn^{56} on chicks with perosis produced by a synthetic manganese deficient diet. Proc. Soc. Exp. Biol. Med., *54:* 197.
9. Hill, R. 1965 The retention of an oral dose of radioactive manganese in the pullet and the effects on retention of the intake of inactive manganese. Brit. J. Nutr., *19:* 171.
10. Hubbell, C. H. 1963 Feedstuff's analysis table for feed ingredients. Feedstuffs, 35(17): 14.
11. Heuser, G. F. 1955 Feeding Poultry, ed. 2. John Wiley and Sons, Incorporated, New York.

Effect of Level and Pattern of Essential Amino Acids on Nitrogen Retention of Adult Man [1]

GLADYS S. ROMO AND HELLEN LINKSWILER
Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin

ABSTRACT Two studies were conducted to determine the effect on nitrogen retention of increasing the dietary intake of essential and semiessential amino acids, patterned as in the poorly balanced protein of corn, when total nitrogen was held constant. Men were fed diets containing approximately 6.00 g nitrogen daily of which crystalline amino acids and diammonium citrate provided 5.50 g and ordinary foods, the remainder. During five experimental periods of study 1, the eight essential and four semiessential amino acids patterned as in corn protein provided 1.10, 2.20, 3.30, 4.40 and 5.50 g nitrogen daily. During the sixth experimental period, 2.20 g nitrogen was also provided by the essential and semiessential amino acids, but the level of isoleucine was increased to that in the FAO provisional pattern. Each increase in the intake of essential and semiessential amino acids was accompanied by an increase in nitrogen retention. Increasing the level of isoleucine to that in the FAO pattern had no effect on nitrogen retention. In the second study, nitrogen balance responses of men fed the corn and egg pattern were compared; equivalent amounts of nitrogen, 2.20, 3.30, or 5.50 g, were supplied by the essential and semiessential amino acids of the two patterns. When the essential nitrogen provided 3.30 or 5.50 g, more highly positive nitrogen balances were obtained when subjects were fed the egg pattern than when they were fed the corn pattern of amino acids.

Usually proteins from animal sources are superior in nutritive value to plant proteins for humans, and this superiority has generally been attributed to the nearly ideal ratio of essential amino acids one to another. Perhaps insufficient attention has been given to the fact that the relative percentage of essential amino acids is higher in animal proteins than in plant proteins. Data from tables of amino acid composition of foods (1, 2) indicate that the eight essential and the four semiessential (arginine, histidine, cystine and tyrosine) amino acids are more than 62% of the nitrogen of egg protein, but only 48% of the nitrogen of corn protein; furthermore, the leucine content of corn protein is very high and accounts for over 20% of the nitrogen contributed by the 12 essential and semiessential amino acids. Some of the nitrogen of both corn and egg is nonprotein nitrogen; approximately 84 and 87%, respectively, of the nitrogen of corn and egg is protein nitrogen. Results of studies by several investigators (3–7) indicate that nitrogen retention by adult humans fed a variety of amino acid patterns is affected by the amount of nitrogen furnished by the essential amino acids. Kies and Linkswiler (8) observed that increasing the amount of dietary essential amino acids patterned as in egg protein, while holding total nitrogen intake constant at 6.0 g daily, resulted in highly significant increases in nitrogen retention in the adult human. Similar results have been reported by Clark et al. (9).

The studies reported in this paper were made to determine whether increasing the dietary intake of the essential amino acids patterned as in the poorly balanced protein of corn would have a beneficial or an adverse effect on nitrogen retention in adult human subjects. During the first of two studies, the effect on nitrogen retention by the adult male of varying the amount of the essential plus semiessential amino acids patterned as in corn protein, while holding total nitrogen intake constant, was observed. In the second study nitrogen balance responses of men were compared when equivalent amounts of nitrogen were supplied by the essential plus

Received for publication July 31, 1968.

[1] Published with approval of the Director of the Wisconsin Agricultural Experimental Station.

TABLE 1

Sources and amounts of dietary nitrogen for each of the experimental diets

Diet no.[1]	EAA and SEAA N [2]	DAC and glycine N [2]	Ordinary food N [3]	Total N intake
	g/day	*g/day*	*g/day*	*g/day*
		Study 1		
C–20	1.10	4.40	0.80	6.30
C–40	2.20	3.30	0.80	6.30
C–60	3.30	2.20	0.80	6.30
C–80	4.40	1.10	0.80	6.30
C–100	5.50	0.00	0.80	6.30
C–40+I	2.20	3.30	0.80	6.30
		Study 2		
C–40	2.20	3.30	0.60	6.10
C–60	3.30	2.20	0.60	6.10
C–100	5.50	0.00	0.60	6.10
E–40	2.20	3.30	0.60	6.10
E–60	3.30	2.20	0.60	6.10
E–100	5.50	0.00	0.60	6.10

[1] The names of the diets indicate the pattern of amino acids (C = corn; E = egg) in the diet and the percentage of nitrogen from purified sources which was supplied by the essential plus semiessential amino acids. I = level of isoleucine as in FAO provisional pattern.

[2] EAA = essential amino acids; SEAA = semiessential amino acids; DAC = diammonium citrate.

[3] Composed of the following: applesauce, peaches, pears, green beans and tomatoes, 100 g each; sugar mints, hard candy, carbonated beverage, butter, oil and jelly, according to individual caloric needs; tea or coffee, constant intake; wafers containing cornstarch, sugar and fat. A mineral supplement supplied the following daily (in grams): Ca, 1.000; P, 1.000; Mg, 0.200; Fe, 0.015; Cu, 0.002; Mn, 0.002; I, 0.00015; and Zn, 0.0009. A vitamin supplement given daily contained 4500 USP units vitamin A and the following (in milligrams): choline dihydrogen citrate, 180; riboflavin, 3.0; thiamine·HCl, 3.0; D-pantothenyl alcohol, 4.5; pyridoxine·HCl, 3.0; niacinamide, 9.0; folic acid, 0.6; biotin, 0.15; vitamin B_{12}, 0.001; *dl*-α-tocopheryl acetate, 4.5; and ascorbic acid, 50.

semiessential amino acids of egg protein and corn protein.

PROCEDURE

Men subjects were fed diets containing approximately 6.00 g nitrogen daily in which crystalline amino acids and diammonium citrate provided 5.50 g of nitrogen, and ordinary foods, the remainder. Study 1 was divided into six 5-day experimental periods, and study 2, into six 4-day periods. Semipurified diets similar to those fed during the experimental periods were given prior to the experimental periods until the subjects' nitrogen output stabilized.

A different level or pattern of essential and semiessential amino acids was fed in each experimental period (table 1). During five experimental periods of study 1, the eight essential and the four semiessential amino acids patterned as in corn protein were fed at five different levels in amounts equal to those present in 18, 37, 55, 73 and 91 g corn protein. These quantities of amino acids furnished 20, 40, 60, 80 and 100%, respectively, of the nitrogen from purified sources and provided 1.10, 2.20, 3.30, 4.40 and 5.50 g nitrogen daily. During the sixth experimental period, 40% of the nitrogen was provided by the essential plus semiessential amino acids, but the level of isoleucine was the same as in the FAO provisional pattern. During study 2, essential and semiessential amino acids patterned as in egg and as in corn (2) were fed in amounts that provided 40, 60 and 100% of the nitrogen from purified sources. During three experimental periods, amino acids patterned as in egg protein provided 2.20, 3.30 and 5.50 g nitrogen daily, and in the other three experimental periods amino acids patterned as in corn protein furnished the same amounts of nitrogen as those furnished by the egg pattern. The essential and semiessential amino acids which provide these amounts of nitrogen are equivalent in amounts to those in 26, 38 and 64 g egg protein or in 37, 55 and 91 g corn protein. Isonitrogenous amounts of glycine and diammonium citrate were added when necessary to maintain the nitrogen intake from purified sources at 5.50 g. Prior to the studies, the amount of calories required for weight maintenance by each individual was esti-

TABLE 2

Age, height, weight and daily caloric intake of the subjects

Subject	Age	Wt		Height	Calorie intake
		Initial	Final		
	years	*kg*	*kg*	*cm*	*kcal/kg body wt*
			Study 1		
1	22	88	85	185	44
2	22	77	76	187	52
3	25	79	77	175	48
4	21	71	70	185	46
5	22	84	85	183	45
6	34	77	76	180	46
			Study 2		
7	26	68	69	176	45
8	23	70	68	179	45
9	26	71	70	190	45
10	35	70	68	182	46
11	35	82	82	187	45
12	22	76	72	174	45

mated. The calorie intake for each individual was then held constant throughout the experimental periods. The calorie intake of the subjects of study 1 ranged from 44 to 52 kcal/kg body weight; that of the subjects of study 2, from 45 to 46.

Random arrangement of experimental diets. To eliminate bias concerning the order in which the diets were given to the subjects, the diets were fed in a randomized sequence. With such an arrangement, no two subjects were fed the diets in the same sequence.

Subjects. The subjects were 12 young men, students at the University of Wisconsin; 6 participated in each study. Information concerning the subjects is presented in table 2. All were considered to be in good health as evidenced by a physician's examination.

Collection and handling of metabolic materials. Complete 24-hour urine collections were made throughout the studies. Fecal composites for each subject were made for each experimental period. Analyses for urinary creatinine (10) and nitrogen (11) were made daily on fresh urine samples. Nitrogen content of amino acid mixtures, other foods and excreta was determined by the boric acid modification of the Kjeldahl method (11). Analysis of variance was used to detect significance of difference in response to the experimental diets.

RESULTS

Mean values for nitrogen balance for each subject and the mean for all subjects during each experimental period of both studies are shown in figures 1 and 2. Nitrogen retention increased significantly with each increase in dietary essential and semiessential amino acids ($P < 0.005$ for study 1; $P < 0.025$ for study 2).

During study 1 the mean nitrogen balances were − 1.23, − 0.68, − 0.22, 0.28 and 0.68 g nitrogen daily when the essential and semiessential amino acids patterned as in corn provided 1.10, 2.20, 3.30, 4.40 and 5.50 g of nitrogen daily, respectively. All six men subjects were in positive balance while consuming the C-100 diet in which the essential plus semiessential amino acids provide 100% of the purified nitrogen (5.50 g), whereas all subjects were in negative balance when receiving either the C-20 or the C-40 diet. As the amount of dietary nitrogen provided by the essential and semiessential amino acids was increased from 1.10 to 5.50 g, the average increment in nitrogen retention was 1.92 g daily.

During study 2 when 40, 60 and 100% (2.20, 3.30 and 5.50 g, respectively) of the purified nitrogen was supplied by the essential and semiessential amino acids patterned as in corn, mean daily nitrogen balances of the subjects were − 0.14, − 0.03 and 0.51 g, respectively; the respec-

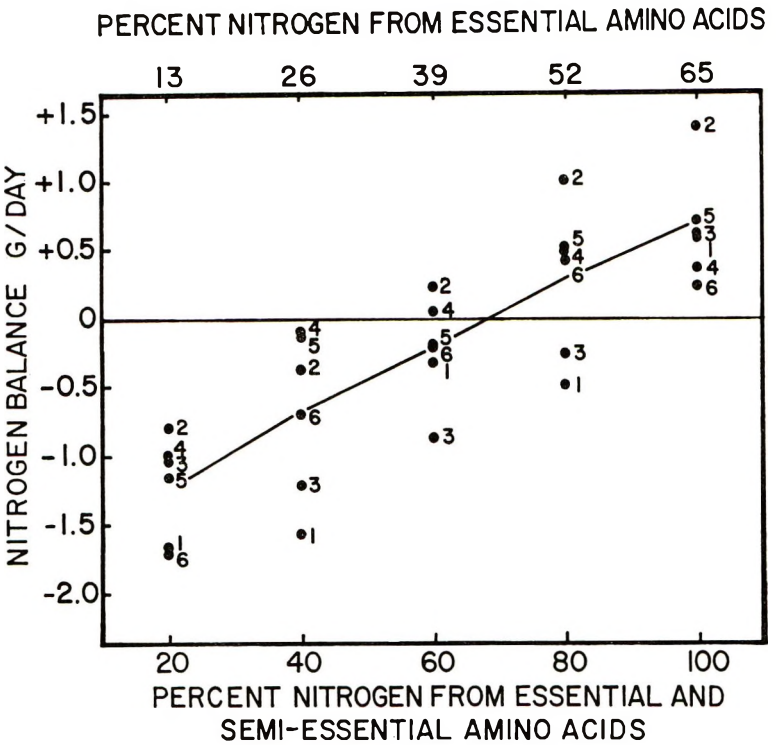


Fig. 1 Effect of varying the intake of the essential and semiessential amino acids patterned as in corn on nitrogen balance of men fed diets containing 6.30 g nitrogen daily. The percentage nitrogen indicated on the abscissa of the graph represents only that furnished by the crystalline amino acids and diammonium citrate and not the 0.80 g furnished by the ordinary foods. The closed circles with numbers refer to the daily nitrogen balance of individual subjects; the line represents the mean daily nitrogen balance for all subjects.

tive mean nitrogen balances of the subjects fed the egg pattern at the same essential and semiessential nitrogen intakes were − 0.26, 0.15 and 0.93 g. Five of the six subjects were retaining nitrogen when the essential and semiessential amino acids patterned either as in the egg or as in the corn protein pattern provided 5.50 g nitrogen daily; but when the essential and semiessential amino acids furnished 2.20 g nitrogen only three subjects fed the corn pattern and two fed the egg pattern were in positive nitrogen balance. Increasing the essential and semiessential nitrogen from 2.20 to 5.50 g resulted in average increments of 0.65 and 1.10 g nitrogen daily, respectively, for subjects fed the corn pattern and the egg pattern. When 5.50 g of essential and semiessential nitrogen was given, subjects retained significantly more nitrogen when fed the egg pattern than when fed the corn pattern ($P < 0.05$). When the essential and semiessential amino acid furnished 2.20 g of nitrogen daily, increasing the level of isoleucine in the corn pattern so that the isoleucine-leucine ratio equalled that of the FAO pattern had no effect on nitrogen retention of the subjects. The C-40 diet contained 4.46 g leucine and 1.60 g isoleucine, whereas the C-40 + I diet contained 4.46 g leucine and 3.91 g isoleucine; the isoleucine-to-leucine ratio was 0.36:1.00 for the C-40 diet and 0.87:100 for the C-40 + I diet.

DISCUSSION

Although the subjects were fed each experimental diet for only 4 or 5 days, the previous diet did not seem to have an effect on the results obtained since there was no significant change in nitrogen balance over the period of time observed. Studies involving longer experimental periods are in progress to determine if the

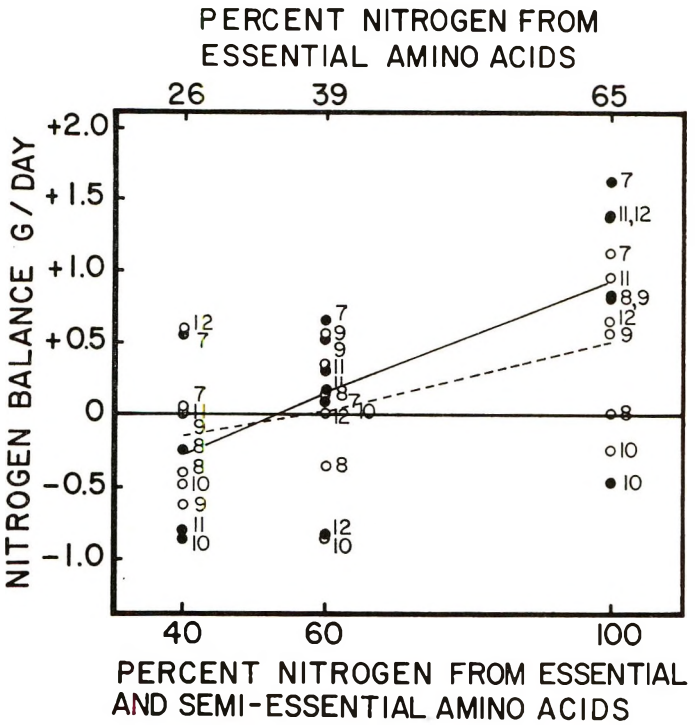


Fig. 2 Effect of varying the intake of the essential and semiessential amino acids patterned as in corn and as in egg on nitrogen balance of men fed diets containing 6.10 g of nitrogen daily. The percentage nitrogen indicated on the graph applies only to that furnished by the crystalline amino acids and diammonium citrate and not the 0.60 g furnished by the ordinary foods of the diet. The open circles with numbers represent the mean daily nitrogen balance of individual subjects for each experimental period when the corn pattern was fed; the closed circles, when the egg pattern was fed. The solid line represents the mean daily nitrogen balance of all subjects when fed the egg pattern; the broken line, the mean daily nitrogen balance of all subjects when fed the corn pattern.

level of essential amino acids has a long-term effect on nitrogen balance when total nitrogen and calorie intake are maintained constant.

When nitrogen intake was approximately 6.0 g, increasing the intakes of the essential and semiessential amino acids patterned after the poorly balanced protein of corn resulted in increases in nitrogen retention and caused changes from marked negative nitrogen balances to distinctly positive ones. The increment in nitrogen retention between the lowest and the highest intake of the essential and semiessential amino acids was 1.92 g daily. When the diet containing 1.10 g essential amino acid nitrogen was fed, only valine, leucine, isoleucine and threonine were present in sufficient quantities to meet the minimum requirement of men for the essential amino acids as established by Rose. But when the diet containing 2.20 g essential nitrogen was fed, the minimum requirement for all the essential amino acids was reached with the exception of tryptophan which met 89% of the minimum requirement. The improvement which resulted when the amount of nitrogen furnished by the essential and semiessential amino acids was raised from 1.10 to 2.20 g may have been due to the increase in the amino acids which are limiting in corn. When, however, tryptophan and lysine were no longer deficient, there was continued improvement in nitrogen balance in response to increases in the intake of the essential and semiessential amino acids. But whether the progressive improvement in nitrogen balance which accompanied the increases in intake of the essential and

semiessential amino acids was caused by increasing the intake of the amino acids which are limiting in corn or by increasing the intake of all cannot be ascertained from this investigation. What is clear, however, is that increasing the dietary intake of the essential amino acids of the poorly balanced protein of corn has a beneficial and not an adverse effect on nitrogen retention in adult human subjects.

The subjects easily achieved a positive nitrogen balance when the essential and semiessential amino acids of the corn pattern contributed 4.40 g or more of nitrogen when the total nitrogen intake was about 6.00 g daily. The data of Kies et al. (12) show that men given 6.0 g nitrogen from corn meal plus an additional 0.7 g nitrogen from the basal diet were unable to maintain nitrogen equilibrium and lost about 0.5 g nitrogen/day; when corn provided 8.0 g nitrogen, the subjects retained about 0.5 g nitrogen daily. Calculations indicate that the subjects of Kies and coworkers were receiving approximately 2.40 and 3.20 g nitrogen from the essential and semiessential amino acids when the respective intakes of nitrogen from corn were 6.0 and 8.0 g. When the subjects of the present study were given 2.20 g nitrogen from the essential and semiessential amino acids of the corn pattern, they lost slightly more than 0.5 g nitrogen daily.

Nitrogen balances of the subjects during the second study were more highly positive when they were fed the egg pattern of essential and semiessential amino acids than when they were fed the corn pattern in amounts which provided 60 or 100% of the nitrogen from purified sources. When the subjects were given 5.50 g essential and semiessential nitrogen, they retained 0.5 g more nitrogen daily when given the egg pattern than when given the corn pattern.

Each increase in the intake of essential and semiessential amino acids of the egg and of the corn pattern by the men was accompanied by an increase in nitrogen retention. These results agree with those of several other investigators. Kirk et al. (3) compared the nitrogen balance response of young women to the FAO provisional reference pattern of amino acids; the food patterns were superior to the FAO pattern. Although the total nitrogen intake and the intake of the amino acid presumed to be limiting in each food were held constant in the comparative studies, the food patterns furnished almost twice as much essential amino acid nitrogen as the FAO pattern. The authors suggested this fact may have been responsible for the superiority of the food patterns. Swendseid et al. (4) fed men diets containing FAO or egg pattern mixtures with the amino acids proportioned to an equal amount of tryptophan for both patterns and found better nitrogen balance was obtained with the egg pattern. When the diets contained isonitrogenous amounts of the amino acids as the FAO or egg pattern, similar nitrogen balance values were found for all subjects. In a subsequent experiment essentially the same results were obtained with women subjects (5). Leverton and Steel (6) reported that amino acids proportioned as in the FAO and oat patterns were equally effective in maintaining approximate nitrogen equilibrium. Oldham and Dickinson (7) combined and analyzed the data from the above four studies (3–6) to further evaluate the nitrogen balance of the adult human fed amino acids proportioned as in the FAO provisional pattern and as in egg, oats, milk and peanuts. The analysis showed a significant regression of nitrogen balance on essential amino acid intake.

Kies and Linkswiler (8) obtained with men subjects an average increment in nitrogen retention of 1.63 g daily when the amount of nitrogen provided by the essential and semiessential amino acids patterned as in egg was increased from 1.31 to 5.23 g with total nitrogen intake at approximately 6.00 g; maximum improvement occurred when the essential and semiessential amino acids contributed 100% of the nitrogen with 62% being furnished by the essential amino acids. In a second study they observed an increment of 1.22 g nitrogen daily when the essential amino acid nitrogen was increased from 1.11 to 5.36 g; nitrogen retention continued to improve as the nitrogen furnished by the essential amino acids was increased from 20 to 80%, and possibly to 100%. Clark et al. (9) reported that simultaneous in-

creases in intake of all essential amino acids caused increased nitrogen retention; mean nitrogen balances were −0.07, 0.22, 0.56, and 0.72 g, respectively, when 1.0, 1.5, 2.0 and 2.5 times the basic mixture was given. The regression of nitrogen balance on essential amino acid intake was linear and did not differ when 6.00 or 9.00 g of total nitrogen was consumed.

The data from this laboratory and from other laboratories as well clearly demonstrate the marked effect that the relative percentage of the essential amino acids has in determining the nutritional value of a protein, particularly when total nitrogen intake is low.

LITERATURE CITED

1. Frost, D. V., and H. R. Sandy 1949 Assay of dry proteins by rat repletion method. Nutritive value and amino acid composition of six reference proteins. J. Nutr., *39:* 427.
2. Orr, M. L., and B. K. Watt 1957 Amino acid content of foods. Home Econ. Res. Rep., no. 4. U.S. Dep. Agr., Washington, D.C.
3. Kirk, M. C., N. Metheny and M. S. Reynolds 1962 Nitrogen balances of young women fed amino acids in the FAO reference pattern, the milk pattern and the peanut pattern. J. Nutr., *77:* 448.
4. Swendseid, M. E., J. H. Watts, C. L. Harris and S. C. Tuttle 1961 An evaluation of the FAO amino acid reference pattern in human nutrition. I. Studies with young men. J. Nutr., *75:* 295.
5. Swendseid, M. E., C. L. Harris and S. G. Tuttle 1962 An evaluation of the FAO amino acid reference pattern in human nutrition. II. Studies with young women. J. Nutr., *77:* 391.
6. Leverton, R. M., and D. Steel 1962 Nitrogen balances of young women fed the FAO reference pattern of amino acids and the oat pattern. J. Nutr., *78:* 10.
7. Oldham, H. G., and F. N. Dickinson 1965 Evaluation of nitrogen balance of young women fed amino acids proportioned as in the FAO provisional pattern and as in egg, oats, milk and peanuts. Amer. J. Clin. Nutr., *17:* 360.
8. Kies, C. V., and H. M. Linkswiler 1965 Effect on nitrogen retention of men of altering the intake of essential amino acids with total nitrogen held constant. J. Nutr., *85:* 139.
9. Clark, H. E., K. Fugate and P. E. Allen 1967 Effect of four multiples of a basic mixture of essential amino acids on nitrogen retention of adult human subjects. Amer. J. Clin. Nutr., *20:* 233.
10. Peters, J. H. 1942 The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter. J. Biol. Chem., *146:* 179.
11. Scales, F. M., and A. P. Harrison 1920 Boric acid modification of the Kjeldahl method for crop and soil analysis. J. Ind. Eng. Chem., *12:* 350.
12. Kies, C. V., E. Williams and H. M. Fox 1965 Determination of first limiting nitrogenous factor in corn protein for nitrogen retention in human adults. J. Nutr., *86:* 350.

letters

EXPERIMENTAL AMYLOIDOSIS IN MICE

The dietary control used by Dr. William West in his studies, "Experimental Amyloidosis in Mice: Effect of high and low protein diets" (J. Nutr., *95:* 323, 1968), is inadequate to prove any difference with changes in protein level.

The high casein diet used had quite a different salt mixture than the low casein diet, as well as containing 2% brewer's yeast. Also, 22% sucrose was used in the high casein diet and none in the low casein diet (and 78% starch was substituted for the casein and sucrose in the latter diet). Unless casein was the only variable in the experiment, I do not see how the results can be attributed to the changes in the casein or protein level.

GEORGE M. BRIGGS
Professor of Nutrition
University of California, Berkeley
Berkeley, California 94720

EFFECTS OF DIETARY DEFICIENCIES OF LIPOTROPIC FACTORS

I wish to comment on the inadequate dietary controls for the inositol and choline deficiency described in the paper by Ibert C. Wells and J. M. Hogan, "Effects of Dietary Deficiencies of Lipotropic Factors on Plasma Cholesterol Esterification and Tissue Cholesterol in Rats," which appeared in J. Nutr., *95:* 55, 1968 (May).

The results with inositol and choline deficiency should not have been included in the paper, I feel, since there was no proper control in these instances by which one can compare results. The so-called inositol deficient diet contained 0.5% succinylsulfathiazole, which in all probability had far greater effects on the results than a "deficiency" of inositol might have given. There was no positive control diet containing the sulfa drug to which inositol was added. (A paper by Leveille and Chakrabarty, on page 88 of the same issue, described changes in cholesterol levels of rats when fed succinylsulfathiazole, which is but one example of the difficulties involved.) A growth rate in male rats of only 3.8 g/day as a result of inositol deficiency, itself, would be most unexpected. Rather, one would expect no growth difference at all, and the term "inositol deficiency" is quite meaningless as used here.

A still greater depression of growth was obtained in the male rats as a result of choline deficiency (the rats grew only 1.4 g/day). In seeking the cause of this depression, one finds that a different diet (diet 2) was fed to the deficient animals. This diet contained 4% cod liver oil which is known usually to be growth-depressing at this level in itself. There were other changes in the dietary ingredients as well. In any event, there were no data presented showing the results with the identical diet plus choline, so again the results need to be completely discounted as far as choline is concerned.

GEORGE M. BRIGGS
Professor of Nutrition
University of California, Berkeley
Berkeley, California 94720

THE AUTHOR'S REPLY:

One cannot but wonder whether or not Professor Briggs' expressed feeling of disfavor might have arisen from a misinterpretation. Although we may not have been successful, we tried very hard to state in the paper that we were studying the effects

Letters may be considered for publication when the writer comments constructively concerning a paper that has been published in the *Journal of Nutrition*. Such letters will be subject to the usual editorial review and at the same time, the author or authors of the paper in question will be given the privilege of submitting a rebuttal. Final acceptance of letters shall be the prerogative of the Editorial Board. (Letters should be as concise as possible and not longer than 500 words including references cited.)

of feeding rats diets which were deficient in the nutrients named. Because in most cases we did not elicit the characteristic symptoms, other than growth depression, exhibited by deficient animals, we considered it unreasonable to claim that the experimental animals were indeed deficient.

Although no control diet containing succinylsulfathiazole was fed, we did not expect this substance to affect cholesterol metabolism. This expectation was fully justified by the work in the reference cited by Professor Briggs. In those experiments it was found that succinylsulfathiazole, when fed in cholesterol-free diets to rats at twice the concentration employed in our experiments, had no effect on liver and plasma lipids and cholesterol. Since our diet was also cholesterol-free, it is apparent that the objection by Professor Briggs to the lack of a succinylsulfathiazole control is not reasonable on a practical basis.

During several years of experience of producing choline deficiency in rats by feeding the diet in question (diet 2), a growth depressing effect attributable to the cod liver oil (850 USP units vitamin A and 85 USP units vitamin D per gram) has not been observed. Rather we find that weanling rats fed this diet, with or without added choline, grow equally well for the first 8 days and at approximately the same rate as weanling rats fed a commercial ration. After the first 8 days, the growth rates of the animals not receiving choline begin to decline. If hemorrhagic kidney degeneration results, the body weights plateau and then begin to decline because the animals essentially cease to eat. It is our impression that this pattern has been observed by many others who have worked in this field.

IBERT C. WELLS
Professor of Biochemistry
The Creighton University
Omaha, Nebraska 68131

The Journal of
NUTRITION
OFFICIAL ORGAN OF THE AMERICAN INSTITUTE OF NUTRITION

A COPYRIGHTED SCIENCE PERIODICAL PUBLISHED MONTHLY BY
THE AMERICAN INSTITUTE OF NUTRITION

Guide for Authors

(Revised January 1969)

Reprints of this Guide for Authors, published in the first issue of each volume of the Journal, are available upon request to the Editor or to the Redactory Service, THE JOURNAL OF NUTRITION, 9650 Rockville Pike, Bethesda, Maryland 20014. It is recommended for the use of authors, secretaries, associates, and students.

THE JOURNAL OF NUTRITION welcomes concise reports of original research bearing on the nutrition of any organism. Preliminary notes, or papers already published or in the press elsewhere are not acceptable. Consideration will be given to the publication of supplements at the author's expense.

In addition to reports of original research, consideration will be given to papers in which new nutritional concepts are developed from information available in the existing nutrition literature.

Letters to the Editor will be considered for publication when the writer comments constructively concerning a paper that has been published in THE JOURNAL OF NUTRITION. Such letters will be subject to the usual editorial review and at the same time, the author or authors of the paper in question will be given the privilege of submitting a rebuttal. Final acceptance of Letters to the Editor shall be the prerogative of the Editorial Board. (Letters and authors' rebuttals must be limited to 500 words, including references cited.)

The American Institute of Nutrition subscribes to the Principles of Laboratory Animal Care developed by the Institute of Laboratory Animal Resources of the National Research Council, and expects that authors who submit manuscripts will have adhered to these principles in their experimental procedures.

ORGANIZATION OF MANUSCRIPTS

The manuscripts should be prepared in complete and finished form, typed double-spaced throughout, including references, footnotes, tables, table footnotes, and figure legends. Papers should be organized in the following sections.

Title page. The following information should be listed on page 1:

1. Complete title of paper in upper and lower case letters.
2. Name or names of authors.
3. Institution or laboratory, with city and state.
4. Shortened form of title (not more than 48 letters and spaces).
5. Address and name of author to whom proof is to be sent.

Titles should be concise to permit effective automatic indexing. They should include the name of the organism studied. The numbering of titles in a series of papers is no longer permitted. If reference to the number of such a paper is desired for a series started previously, this may be placed

in a footnote. Titles not meeting these specifications will be reworded in the Editorial Office, subject to the author's approval.

All footnotes, including those pertaining to the title page, should be placed on a separate sheet, typed double-spaced.

Abstract. An abstract of 200 words or less, typed as a single paragraph on a separate sheet and double-spaced, should be included as page 2. Abstracts should state the objective of the study and give a concise description of the plan or design. New observations or methods should be pointed out, with results and conclusions stated clearly. An abstract should be intelligible by itself.

Text. The text should begin on page 3. Use care to make the text readable, clear, and concise. It should be written so as to be understandable in itself, without reference to tables or figures. Use the active voice whenever possible. The text is usually divided into sections, with centered uppercase headings, in the following sequence: Introduction (without heading); Methods, Procedures, or Materials and Methods; Results; Discussion (or these two sections combined as Results and Discussion); and Acknowledgments. This arrangement is not binding on the author if another approach is more suitable.

The words "et al.," "per se" and "ad libitum" are not underscored. In footnotes giving details of composition of diets, the repeated use of a unit may be avoided by stating the common unit used, as "the diet consisted of the following: (in grams) sucrose, 50; casein, 130; . . ." or "8, 12 and 14%." The expression "fed a diet" is preferred to "on a diet" or "given a diet." Certain other preferred usage is described in the *Style Manual for Biological Journals*.[1] Avoid the use of laboratory jargon and such phrases as "it can be seen that," "it is interesting that," and "it can be noted that."

Numbers: Use arabic numerals, not Roman or ordinal numerals, throughout, including those in tables and figures (e.g., group 3, not the third group or group III; table 1, not table I). If possible, avoid beginning a sentence with a numeral or a symbol.

Statistical methods: Tests of statistical significance should be identified, and references used should be cited. Statements that results are statistically significant should be accompanied (in text, tables, and figure legends) by indications of the level of significance (as P, the probability of the event's being due to chance alone, a decimal quantity; or as α, the level of significance, a percentage).

Units of measure, abbreviations, symbols, and nomenclature: The metric system is used for all units, and temperature is expressed in the centigrade scale (degree sign without C). Letters in abbreviations such as NAD and IU are not spaced; periods are omitted except when the abbreviation might be read as another word. *Do not* add "s" when the plural is meant. Following is a list of the more common abbreviations and symbols used in the Journal:

average	avg (*tables only*)
calorie ("small," gram calorie)	cal
Calorie ("large," kilogram calorie)	*not used; use* kcal
centimeter	cm
counts per minute	cpm
cubic millimeter	mm^3
degree	° (*omit* C)
degrees of freedom	df (*tables only*)
gram	g
hour	hr (*tables only*)
international unit	IU
kilocalorie	kcal
kilogram	kg
liter	(*spell out*)
meter	m
microcurie	μCi
microgram	μg (not γ)
microliter	μl (not λ)
micromicrogram	picogram, pg (preferred to $\mu\mu$g)
micron (10^{-6} meter)	μ
micromolar (concentration)	μM
micromole (mass)	μmole (never μM)
millicurie	mCi
milligram	mg
milligrams %	(*never use; use* mg/100 mg, mg/100 ml, *or* mg/100 g, *as appropriate*)
milliliter	ml (*not* cm^3 *or* cc)
millimeter	mm
millimicrogram	nanogram, ng (preferred to mμg)

[1] Conference of Biological Editors, Committee on Form and Style 1964 Style Manual for Biological Journals, ed. 2. American Institute of Biological Sciences, Washington, D.C.

millimicron (10^{-9} meter)	mμ
millimole	mmole
minute	min (*tables only*)
molar (moles per liter)	M
mole	mole (*never* M)
nanogram (10^{-9} g)	ng
parts per million	ppm
percent	%
picogram (10^{-12} g)	pg
probability (statistics)	P
second	sec (*tables only*)
square centimeter	cm^2
square meter	m^2
square millimeter	mm^2
standard deviation	SD
standard error	SE
standard error of the mean	SEM
t (statistics)	*t*
weight	wt (*tables only*)
year	yr (*tables only*)

Other commonly accepted abbreviations may be found in the *Style Manual for Biological Journals*. In general, chemical and biochemical terms and abbreviations should conform with the usage recommended by the International Union of Pure and Applied Chemistry (IUPAC) and its committees on nomenclature. Names of chemicals are written out in the text; formulas may be used in tables or figures, or both. When appropriate, identify enzymes with their EC number and systematic name.[2]

Isotope specification should conform to the IUPAC system, with the mass number placed as a superscript preceding the chemical symbol, as ^{14}C, *not* C^{14}.

Acknowledgments. Financial support should be listed in a footnote to the title. Credit for materials should be listed as footnotes in the text. Technical assistance and advice may be cited in a section headed Acknowledgments, which will appear at the end of the text.

Literature cited. Accuracy and adequacy of the references are the responsibility of the author. Literature cited should be checked carefully with the original publication. List references in the order of citation in the text. Be sure *all* references in Literature Cited have been mentioned in the text (or tables or figures). References to a paper in press may be included in Literature Cited. If such a paper would be useful in the evaluation of the manuscript under consideration, it is advisable to make a copy of it available to the Editor. When a manuscript is one of a series of papers, the preceding paper should be included in Literature Cited. References to abstracts of verbal reports and to other unedited material (as the abstracts in Federation Proceedings, theses, and industrial technical bulletins) should be treated as text footnotes. Personal communications and unpublished experiments should be treated as footnotes to the text.

Follow the style in recent issues of the Journal for references. Do not underscore titles of journals or books; do underscore volume numbers of journals. All references should be double-spaced. Names of journals should be abbreviated in accordance with the list of abbreviations in the *Style Manual for Biological Journals*.

Footnotes. In the text, footnotes should be numbered consecutively, including any indicated on the title page. For tables, footnotes should be typed double-spaced directly beneath the table, and numbered 1, 2, 3, etc. Superscripts in the table should appear consecutively, starting at the top of the table and reading from left to right across each line. Footnotes to tables are independent of footnotes to the text.

Footnotes are used for the following types of information:

1. Address(es) of author(s) if different from the institution or laboratory given on page 1. Include zip code number.
2. Approval of paper for publication.
3. Acknowledgment of support of research.
4. Prior presentation at meetings or symposia, with name, location and date of presentation. If an abstract was published, a citation should also appear in this footnote.
5. Name and address for reprint requests, if not the first author and address stated on page 1.
6. Source of materials, feeds, instruments, etc. (name, model number, manufacturer's name and location (city and state)).
7. Source of laboratory animals (same information).
8. Composition of diets, or vitamin or mineral mixtures, if not presented in a table.
9. References to abstracts, verbal reports or other unedited material.
10. Personal communications and unpublished experiments, including names of information sources and year.

[2] The International Union of Biochemistry Commission of Editors of Biochemical Journals 1965 Enzyme Nomenclature. Elsevier Publishing Company, Amsterdam, London and New York.

Registered trade names are not used in text, tables, or figures. A trivial or descriptive name should be used in text and tables, with a footnote giving the registered trade name, manufacturer and address (city and state).

Tables. Follow form in current issues for the use of upper and lower case letters and italics. Do not use vertical rules. Units of measure should be indicated clearly two spaces above the first value in a column. Tables and their footnotes (numbered independently from those in the text) should be typed double-spaced; if this requires the use of more than one page per table, simply indicate "cont'd" on the first page and "Table X, cont'd" on the following page(s). A table or figure should be constructed so as to be intelligible without reference to the text. Lengthy tabulation of essentially similar data can often be avoided by giving the number of experimental results and their mean values, with standard deviations, standard errors or ranges within which the values fall. Statements that significant differences exist between the mean values of two groups of data should be accompanied by indications of probability derived from the test of significance applied. Tables should be numbered (table 1, table 2, etc.) in order of appearance in text; references cited in table should be numbered according to the location of the table in the text. Authors are urged to economize on space used for tables and figures. These should fit one column width (2⅝ inches) or, when necessary, two column widths (5½ inches).

Figures. Original drawings, with two reproductions to be sent to reviewers, or in the case of photographs, the original and two glossy prints, should accompany the manuscript. They should be marked on the back in ink or pencil — not ballpoint pen — with the author's name, complete address, and with the figure numbers. They should not be glued, stapled or clipped. The top should be indicated to avoid confusion. Such drawings and photographs must not exceed 8½ × 11 inches in size and must be at least 5¼ inches wide in order to fit the 2⅝-inch single column width when reduced by one-half. When a complicated figure requires more space for clarity, a proportionately larger illustration will be acceptable. But two copies of *prints* should be submitted on sheets of the same size as the text. Oversize or undersize figures are difficult to handle in editing.

Drawings should be on white or blue-white paper or bristol board — not cream-white. They should be prepared for reproduction as line or halftone engravings. Abbreviations, units, and symbols in figures and legends should conform to Journal style. Letters and numbers should be uniform and large enough so that no character will be less than 2 mm high after reduction. A line 0.4 mm wide reproduces satisfactorily when reduced by one-half.

Figures should be lettered (preferably by stencil) in black India ink. For any charts made on cross section paper, use India ink on paper printed in light blue only. Charts drawn in India ink should be so executed throughout, with no typewritten material included. Graphs and charts should be given consecutive figure numbers as they will appear in the text. Legends (including any keys to symbols or charts) should be numbered consecutively and combined on a separate sheet, typed double-spaced.

For the reproduction of any illustration(s) in color, the author must have funds available for full payment and must make advance arrangements with the publisher.

ASSEMBLY OF MANUSCRIPTS

Manuscripts are to be typewritten on bond paper (8½ × 11 inches). Double spacing must be used throughout, including the title page, tables, legends, footnotes, and references. A margin of about 1¼ inches (or 3 cm) is needed at each side of the sheets.

Follow the style of a recent issue of the Journal for general format. *Never divide or hyphenate a word at the end of a line.* Do not staple together the pages of the manuscript.

Start each of the following sections on a new page: 1) title page; 2) abstract; 3) text; 4) literature cited, arranged in the order of citation in the text; 5) footnotes; 6) figure legends, numbered and listed consecutively, each legend as a new

paragraph; 7) tables with appropriate headings, a separate sheet for each table, with headings, data, and footnotes all double-spaced.

Number all pages at top center of page consecutively in the following order: title page, abstract, text, literature cited, footnotes, figure legends, and tables. Assemble in this order, placing figures and illustrations (not to be given page numbers) at the end.

Auxiliary publication: To conserve space in the Journal, unusually lengthy descriptions of experimental procedures, detailed data such as those on individual animals or subjects, extra figures and other material considered important supplementary information, may be deposited without charge to the author with the American Society for Information Science (formerly the American Documentation Institute), Library of Congress, through the Editor's Office, with a footnote reference. This footnote gives information on the availability of photostat or microfilm copies at moderate cost.

SUBMISSION OF MANUSCRIPTS

An original and two copies (legible carbons or photocopies) of the manuscript and abstract should be sent flat by registered mail to

DR. RICHARD H. BARNES
GRADUATE SCHOOL OF NUTRITION
CORNELL UNIVERSITY, SAVAGE HALL
ITHACA, NEW YORK 14850

After the manuscript is reviewed and possible revisions made, it is processed before release to the printer by the Redactory Service of THE JOURNAL OF NUTRITION. If any minor clarification is needed, editorial queries are noted on page 1 of the manuscript. They should be carefully answered at the time the author reads proof. Since authors receive *page* proof of their articles, only essential changes can be made; authors are charged for changes from manuscript. Corrections should be made in regular pencil only, using standard proof reader's marks. *Proof must be returned promptly or publication will be delayed.*

CHARGES TO AUTHORS

Excess illustrative material. A charge of $40 per page will be made by the publisher for that space used for tables and figures which exceeds one-quarter of the space used for the manuscript exclusive of tables and figures.

Page charges. THE JOURNAL OF NUTRITION levies a charge of $30.00 per page. Authors will be billed after they receive page proof. Ability to pay this charge will *in no way* prejudice the acceptance of the manuscript. Charges will be invoiced directly by the business office; the editor's office is not advised concerning the author's payment or nonpayment of the charge.

Reprints. Reprint order forms and rates will be sent with page proof. The amount paid in page charges will be credited against the cost of reprints. Anyone other than an author desiring reprints must have orders approved by the author, and give written assurance that 1) distribution will be restricted to those interested in the science of nutrition, and 2) no advertising or imprinting will be attached to the reprints thus furnished.

THE JOURNAL OF NUTRITION
9650 Rockville Pike
Bethesda, Maryland 20014

ERRATUM

Chakrabarty, Krishna, and Gilbert A. Leveille 1968 Influence of periodicity of eating on the activity of various enzymes in adipose tissue, liver and muscle of the rat. J. Nutr., *96:* 76–82. In tables 1, 2, and 3, pages 78 and 79, headings immediately above columns of data should read *milliunits/mg protein* ^{2} instead of *units/mg protein* ^{2}.