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Francis Gano Benedict

— A Biographical Sketch

(1870–1957)

For his accomplishments over a period of some 40 years Francis Gano Benedict became recognized as one of the world's foremost investigators in the field of respiratory metabolism. He achieved this eminence through a thorough formal training in science, an early association with the pioneer American worker in the field and a genius for devising equipment and methods which he used most effectively in solving a wide variety of problems. He was born October 3, 1870 in Milwaukee, Wisconsin. When he was seven years old his family moved to Florida and four years later to Boston. Here he received his high school education, studied chemistry for one year at the Massachusetts College of Pharmacy and then entered Harvard University where he obtained the A.B. degree in 1893 and the A.M. degree in 1894. He continued his graduate studies at Heidelberg, chiefly under Victor Meyer, and obtained the Ph.D. degree *Magna cum laude* in 1895. His dissertation was entitled "Ueber die Jodoniumbasen aus *p*-Bromjodbenzol."

All of Benedict's training had been in the field of chemistry. Upon his return to the United States in 1895 he received an appointment as research assistant in the Department of Chemistry at Wesleyan University, Middletown, Connecticut, under W. O. Atwater, and here he was introduced to the field of physiology, particularly energy metabolism which became his life work. He was appointed instructor in 1905 and professor in 1907. At the outset he also received an appointment as physiological chemist in the U. S. Department of Agriculture since Atwater was in charge of studies on problems of human nutrition for which funds were annually appropriated to the Department. A later appointment (1896) was as chemist of the nearby Storrs Agricultural Experiment Station of which Atwater was Director. Benedict remained at Wesleyan until Atwater's death in 1907, and then was appointed Director of the Nutrition Laboratory in Boston, newly established by the Carnegie Institution of Washington — a post which he held until he retired in 1937. Thus, his research career is properly divided into a Wesleyan period of 12 years and a Boston period of 30 years.

In 1897 Benedict married Cornelia Golay of Brewster, Maine, a Vassar graduate who majored in biology. This training enabled her to share with her husband in the conduct of several physiological studies in the Nutrition Laboratory which were published jointly.

In joining the staff of the Department of Chemistry at Wesleyan as an assistant to W. O. Atwater, Benedict became associated with one of the outstanding investigators of his time in the fields of agricultural and physiological chemistry and the founder of the science of nutrition in the United States. (A biographical sketch of Atwater has been published in this Journal, 1962, *78:* 3–9.) Most of Benedict's research at Wesleyan dealt with studies carried out jointly with Atwater using the Atwater-Rosa respiration calorimeter, the construction of which had been initiated in 1892. At the time of his arrival the calorimetric feature had not been completed and thus the first results reported were obtained by indirect calorimetry through the measurement of nitrogen-carbon balances. In 1897 direct calorimetric studies were begun and a total of some 500 experiments were carried out during the next ten years. They dealt with the quantities of nutrients and energy metabolized under different conditions of rest and muscular activity, the relations between external work and the energy and nutrients metabolized in its performance, the capacity of different classes of nutrients to supply the body with

nutrients and energy and the proportions in which they may replace each other for these purposes. The results were published under the joint authorship of Atwater and Benedict and several collaborators in six bulletins of the Office of Experiment Stations of the U. S. Department of Agriculture, under the general title "Experiments on the metabolism of matter and energy in the human body."

A series of studies which attracted wide popular as well as scientific attention dealt with the physiological action of alcohol, as reported in a Memoir of the National Academy of Sciences in 1909. It was found that alcohol could provide energy for warmth and probably for work and could protect body tissues from catabolism. The results of these studies brought a storm of criticism from temperance organizations, both of the investigators and of Wesleyan University, then a Methodist institution. To combat the furor thus created Atwater wrote articles in various popular journals explaining the findings, emphasizing that the quantity of alcohol effectively used was small, that it could have deleterious effects as well, and advocating a rational method of temperance reform. Benedict discussed the significance of the findings in medical journals. Later at Boston, he continued studies of the physiological and psychological effects of moderate doses of alcohol, with various collaborators, both before and after the beginning of the Prohibition era. Though he was a strong foe of alcohol in any form throughout his life, this fact did not interfere with the objectivity of his studies.

Benedict's last calorimetric study at Wesleyan was carried out jointly with Thorne M. Carpenter who later became his associate throughout the Boston era. This study dealt with the effect of muscular and mental work on metabolism.

The respiration calorimeter provided for the indirect determination of oxygen. Atwater wanted to design an apparatus for its direct determination and in 1902 obtained a grant from the Carnegie Corporation for the purpose. The task of designing and construction of such an apparatus became Benedict's responsibility and he brought it to a successful completion in 1904. The apparatus and its functioning were described in detail in a monograph of 193 pages published by the Carnegie Institution of Washington in 1905. In 1924 Benedict referred to this accomplishment as his first important contribution to science.

In addition to his investigations carried out in cooperation with Atwater, Benedict conducted independent research in methods of organic analysis, nitrogen metabolism, and factors causing body temperature variation, which resulted in some 15 publications. He also published a short article in *Science* on the teaching of chemistry in grade and secondary schools.

Atwater's laboratory at Wesleyan became very crowded as his program expanded, as well as having other disadvantages. About 1903 the Carnegie Corporation had agreed to build a new laboratory for Atwater at Wesleyan and endow his program. Shortly after this time, however, it became apparent that he would be permanently incapacitated by illness and thus the proposed support was transferred to the establishment of a Nutrition Laboratory in Boston of which Benedict became Director. A major reason for this shift from Middletown was to have a location where clinical studies could be carried out in cooperation with medical investigators. It had become recognized by certain medical scientists, notably Dr. W. H. Welch, that the studies reported from Wesleyan were of much significance in medicine. Boston was chosen because it had large hospitals and medical schools.

While the laboratory was under construction Benedict made a trip to Europe in early 1907 to study methods and equipment and ideas for the program he would inaugurate in Boston. While there he lectured in German and French at various universities. He repeated these trips frequently in later years. The laboratory building was completed in 1908 and actual work on the construction and testing of apparatus begun.

In 1910 Benedict stated that the Laboratory in Boston was established to provide for a study of the nutrition of man, as a direct outcome of a series of investigations originally undertaken by Atwater. Thus, initially, his primary purpose was to continue calorimetric investigations. As time went on these investigations led him to make correlated studies in other fields. At the outset he and his technical assistants

built two "small" respiration calorimeters patterned after the Atwater-Rosa apparatus, but only one-third its size — one containing a chair, the other a bed. As Benedict's program proceeded, other respiration calorimeters were built for special purposes as well as respiration chambers without the calorimetric feature. In addition, he developed various simple respiration devices for studies with man and various animals. One such was the Benedict apparatus which was widely used in hospitals and doctors' offices for measuring the metabolism of patients. He also built an oxy-calorimeter to determine the gross energy value of foods, excreta and fuels. Benedict had a genius for devising special apparatus to meet his research objectives and in so doing provided tools which were used by many other physiologists. This was one of his very important contributions to the advancement of scientific research.

At the outset, studies were begun with the respiration calorimeter, with Thorne M. Carpenter as his chief associate. These studies dealt with respiratory exchange and heat production under a variety of conditions such as fasting, undernutrition, the ingestion of specific nutrients, fever, mental activity, rest, muscular activity and others. Respiration chambers and simpler devices were used for many specific studies. A study was made with Talbot of the metabolism of the newborn infant and of children up to the time of puberty. For several years a cooperative study was made with C. P. Joslin and associates on the metabolism of diabetes in man, one of the most important series of investigations ever made of this disease.

In 1918 Benedict joined with Professor E. G. Ritzman of the University of New Hampshire at Durham in devising and constructing a respiration apparatus large enough for studies with cattle. Here the investigators drew on the experience of H. P. Armsby who had constructed and operated for some ten years, at Pennsylvania State College, a respiration calorimeter for farm animals. Armsby visited Durham to advise on the progress of the work. This New Hampshire apparatus for large farm animals was the first of its kind in the United States and became the forerunner of others constructed elsewhere for indirect calorimetry. The first study made dealt with undernutrition in steers. The results showed that animals which suffered heavy losses in weight during the winter on submaintenance rations could be restored to satisfactory market quality by fattening rations. This initial study was followed by ten others with cattle, sheep and swine over a period of some 15 years. In 1938 Benedict and Ritzman published a 200-page monograph on the physiology of the adult ruminant.

Benedict's list of publications contains over 30 dealing with studies of the basal metabolism of man. Several dealt with the conditions required to establish the basal state. Many others were concerned with the effect of age, sex, body size, race and other factors. Various races were studied in their own environments. In 1919 Harris and Benedict published standards based on height, weight, age and sex which were widely used in hospitals and doctors' offices for comparing the metabolism of patients with that of normal persons.

Benedict's studies on basal metabolism in man were accompanied and followed by similar studies with a variety of species of animals, reported in a score or more of publications. The investigations included mammals both domestic and wild, ranging in size from the 8-gram dwarf mouse to a 4,000-pound elephant. The reptiles studied included a python, alligators, lizards and tortoises. Several species and races of birds were also included in the investigations. For several of the species Benedict had to design special equipment, either respiration chambers or some type of face mask. His publications contain interesting pictures of the equipment used. The publication reporting his studies with the elephant (Carnegie Institution Monogr. no. 474) gives diagrams of both a respiration chamber and a "trunk breathing apparatus." The studies on wild animals were carried out cooperatively at various institutions — the New York Zoological Park, the Cold Spring Harbor Station of the Carnegie Institution, the headquarters of the Ringling Brothers Circus in Florida and the Yale Anthropoid Experiment Station in Florida.

Upon the basis of his investigations with man Benedict concluded that properly determined basal values were constants, apart from the influence of emotional factors. As a result of his later studies with animals he

recognized that this conclusion did not apply to all species. He referred to the "lability of basal metabolism," found in some species, particularly in the steer, dairy cow and sheep as studied with Ritzman. Clearly there are difficulties in arriving at the basal state in certain animals as compared with man. Ruminants present a special problem because of the long time required to achieve a truly postabsorptive state. Benedict mentioned that the wide degree of variability found in the dairy cow was likely due to genetic and possibly endocrinal factors.

Most of Benedict's data on basal metabolism were expressed per unit of body weight, though in a few publications they were also expressed per unit of body surface calculated from weight, or per weight to the two-thirds power. He considered surface area as a base as advocated by Rubner in his surface area law. He developed a photographic method for measuring surface area and used it to study the relations of body surface to heat production. On the basis of his studies and those of others Benedict concluded that surface area might be useful in estimating normal metabolism, but that it was not causal. He was in disagreement with some American colleagues as to the significance of the surface area measure and particularly with Rubner with whom he had a long controversy in personal correspondence. While continuing to hold his view that the surface area law was quite unsatisfactory, he did pay tribute in 1938 to Rubner's concept in the following words: "Any concept that will have strong adherents after fifty years, any concept that has stimulated as much thought-provoking discussion, and any concept that has required as massive a collection of experimental evidence to contradict it as has Rubner's 'law,' has rendered an incalculable service not only to physiology but to experimental science as a whole."

In his last monograph, entitled "Vital Energetics, a Study in Comparative Basal Metabolism" (Carnegie Institution Monogr. no. 503, 1938), Benedict summarized his studies with both man and the various species of animals, interpreted the meaning of the results and made suggestions for their use. He discussed the basis for intraspecies and interspecies comparisons, and tables illustrating such comparisons were presented. The story indicates that during his later years he emphasized the diversity and inequality of basal metabolism in various species and its lability in some. He concluded the monograph with a discussion of possibilities as to why such differences in total heat production and in the metabolism exist in different species and expressed the hope that his studies might "stimulate search for the true explanation of these striking differences in the level and intensity of vital energetics."

Around 1915 Benedict began studies of the physiology of the goose, which covered two decades. His principal collaborator at the start was Edward L. Fox. Several others participated later including Robert C. Lee who was the joint author of a monograph reporting the various studies, published in 1937. Benedict's primary reason for undertaking these studies was to investigate the formation of fat from carbohydrate. The goose was chosen because of the knowledge that this bird can be made to deposit very large amounts of fat, particularly in the liver and subcutaneous tissue, by forced-feeding, without distress or illness — the practice which produces the "Strasbourg goose." The gaseous metabolism studies made with these forced-fed birds, in which most of the RQ's ranged from 1.3 to 1.4, are of special interest for comparison with the correlated studies made in fasting and in normal feeding.

Over the years of the Laboratory's existence, several workers from foreign countries spent the greater part of an academic year there, supported by grants from it or other institutions. Many other workers came for periods of varying length to become acquainted with the apparatus and methods being used. The work of the Laboratory was concerned almost exclusively with energy metabolism. Protein metabolism received attention in a few of the studies, as did also food composition and digestibility. Surprisingly, while Benedict's years of research paralleled the period when the exciting discoveries with vitamins and amino acids were being made, these new fields of nutrition received no attention from him. Although he was certainly aware of them, he apparently felt that his resources could be used to best advantage in advancing the knowledge of energy me-

tabolism which was basic to all other nutritional processes.

The foregoing account presents examples which highlight Benedict's accomplishments. Certainly it is a limited discussion of experimental results presented in some 400 publications, comprising U.S.D.A. bulletins, monographs of the Carnegie Institution ranging up to 700 pages in length and reports in various United States and foreign journals. Over 90% of these publications reported work carried out at the Nutrition Laboratory, involving a large number of collaborators, only a few of whom have been mentioned here. In a summary publication in 1938 he refers to them as "innumerable." Some mention has been made of workers in other laboratories with whom Benedict collaborated. Additional ones were: Oscar L. Riddle and Morris Steggarda at the Cold Spring Harbor Station; Eugene DuBois of the Cornell Medical College; Carey D. Miller of the University of Hawaii; Lafayette B. Mendel of Yale; H. C. Sherman and Grace McLeod of Columbia, and others. Many of Benedict's papers were written by collaborators but he prepared the first drafts, at least, of a very large number of them. It was his frequent practice to dictate papers to a stenographer or dictaphone, a practice which tended to make the papers too long, but it gave other scientists in the field a complete picture of his results and thoughts regarding them.

Benedict received many honors. He was elected to the American Philosophical Society in 1910, the National Academy of Sciences in 1914, and the American Academy of Arts and Sciences in 1930. He received the medal of the National Institute of Social Sciences in 1917 in recognition of his valuable contributions to human energetics and the alcohol problem. In 1929 the University of Hamburg awarded him a gold medal "in recognition of his successful work in metabolism and human physiology." He was elected to honorary membership in several scientific or medical societies in Europe. The honorary Sc.D. was conferred on him by Wesleyan University in 1910 and by the University of Maine in 1924. He received an honorary M.D. degree from the University of Würzburg in 1932.

The writer remembers Benedict as a large man with an impressive and dignified bearing. Yet he was a very friendly person and an interesting conversationalist. He has mentioned his hobbies as being magic and piano playing. His qualifications in the field of magic are indicated by his election to membership in the Society of American Magicians. In lectures, Benedict's art of showmanship enabled him to present his story in a very interesting manner, illustrated with lantern slides and anecdotes. Both before and after he retired he went on tours throughout the country where he lectured at various universities, on such topics as The Physiology of the Elephant, Magic and Science, Animal Metabolism from Mouse to Elephant.

Upon his retirement from the Nutrition Laboratory in November 1937, Benedict took up his year-round abode at Machiasport, Maine, where he had long owned a summer home. He continued an extensive correspondence with colleagues both at home and abroad, went on several lecture tours and became interested in local affairs. In April 1940 he was hit by a taxi in Boston which resulted in almost fatal injuries. He became much less active thereafter and frequently spent the winter months in warmer climates. Benedict died in Machiasport on May 14, 1957. So ended the career of a man whose active life had been characterized by a steady flow of results from excellently planned and meticulously carried out experiments and who had played a leading role in the development of both basic and applied physiology.

Upon the retirement of Benedict, Thorne Carpenter was appointed Director of the Laboratory. With the onset of World War II, the staff, now depleted by resignations, became almost exclusively engaged in war research. In 1945 Carpenter retired as Director and arrangements were made for the sale of the real estate and buildings of the Laboratory to the Children's Hospital in Boston. Much of the equipment was donated to various scientific and educational organizations. According to the annual report of the President of the Carnegie Institution, the discontinuance of the Laboratory was "in pursuance of a policy which research organizations must follow of re-

linquishing work which they have carried on when the time comes that other organizations are prepared to continue it." A very comprehensive summary of the activities of the Laboratory from its establishment to its close is given by Carpenter, who was a member of the staff throughout its existence, in the Yearbook of the Carnegie Institution, no. 44, 1944 — 1945, pp. 149 to 153.

A short biography of Benedict by Eugene DuBois and Oscar Riddle which lists some 300 of his publications is to be found in Biographical Memoirs, Nat. Acad. Sci. XXXII, 67–78, 1958.

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Partial Dietary Replacement of Milk Protein by Nonspecific Nitrogen in Young Men [1,2]

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ABSTRACT Skim milk protein was replaced isonitrogenously by glycine and diammonium citrate, or by a mixture of nonessential amino acids in the test diet of 21 male college students; the effect of this replacement on urinary nitrogen and sulfur excretion and on fasting plasma amino acid concentration was studied. The subjects were fed a constant nitrogen intake equivalent to 0.38 g protein/kg body weight. Skim milk protein furnished 90% of the daily protein; oatmeal furnished the remainder. In experiments 1 and 2, glycine and diammonium citrate replaced 20 and 25% of total dietary nitrogen, respectively. In experiment 3, a mixture of nonessential amino acids replaced 25% of dietary nitrogen. The 20% replacement with glycine and diammonium citrate did not increase urinary nitrogen excretion in any of the seven subjects, but three of the seven showed lower urinary nitrogen excretion after return to the basal diet. A 25% replacement with glycine and diammonium citrate increased urinary nitrogen excretion in three of seven subjects and four subjects showed lower nitrogen output during the final basal period. The 25% replacement with nonessential amino acids did not change urinary nitrogen excretion but 3 of 10 subjects showed a variable decrease in nitrogen excretion during the second basal period. As a replacement for 25% of total dietary protein, nonessential amino acids appeared a more effective nonspecific nitrogen source than glycine and diammonium citrate. Fasting plasma amino acids levels were unchanged during the experimental periods.

Most of the work done on human protein requirements has dealt with the essential or indispensable amino acids. But the other component of the protein requirement, nonspecific nitrogen (1–4), which is furnished from excess essential amino acids and by the nonessential amino acids (NEAA), merits further investigation. A review of the published values for the essential amino acid requirements of adult man (2, 5, 6) suggests that the concentrations of essential amino acids per gram of total nitrogen in high quality protein foods such as eggs, meat and milk are higher than required when these foods are the sole source of dietary protein. Moreover, a number of investigators have shown in experimental animals that nonessential amino acids are more effective in meeting nonspecific nitrogen requirements than are the essential amino acids (7, 8). To investigate further the interrelationship between these two components of man's protein needs, we have undertaken a series of studies in which various amounts of the test dietary protein are replaced, isonitrogenously, by a source of nonspecific nitrogen. The present work with milk protein extends our previous observations made with egg (9) and beef protein (10).

In establishing the amino acid requirements of man, investigators have used various sources of supplemental nitrogen including glycine or urea (11), glycine in combination with diammonium citrate and other nonessential amino acids (12, 13), or a mixture of several nonessential amino acids (14). Although these studies suggest that a number of individual nonessential amino acids can meet the nonspecific nitrogen needs as effectively as a mixture of nonessential amino acids, the issue is far from settled (15). In the present series of experiments, we have isonitrogenously replaced milk protein first

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by a mixture of glycine and diammonium citrate, and then by a mixture of nonessential amino acids patterned as in cow's milk protein, and compared the effects.

MATERIALS AND METHODS

Subjects. The 21 male college students (table 1) who participated in these studies were in good health as determined by medical history and examination, and continued their normal daily activities throughout the experimental period. Three of the subjects (WI, KE and JR) participated in more than one experiment.

Diet. The composition of the diet, based principally on a liquid formula, is shown in table 2. Dried nonfat skim milk protein supplied 90% of the total dietary protein; oatmeal supplied the remainder. It was found necessary to include a small amount of oatmeal to maintain a stable formula of agreeable consistency. The diet furnished 0.38 g protein/kg per day. The net protein utilization (NPU) of the skim milk protein by rat assay (16) was determined to be 71 ± 2 compared with 63 ± 2 for unsupplemented casein used as a standard. The daily diet was divided into four approximately equal portions.

"Dilution" of the dietary protein. As in our previous studies (9, 10), the dietary nitrogen was replaced ("diluted") by substituting part of the dietary nitrogen with either a mixture of glycine and diammonium citrate (in which each compound supplied equal amounts of nitrogen) or a mixture of nonessential L-amino acids based upon the proportions of amino acids in whole dried nonfat skim milk protein (17). The composition of this mixture is shown in table 3.

Experimental sequence. The subjects were first fed the basal (0% dilution) diet which supplied 0.38 g protein/kg body weight. This rate was calculated to supply nitrogen at the minimum daily nitrogen requirement as discussed previously (9, 10). The duration of basal period 1 varied

TABLE 1

Age, height, weight and calorie intakes of young men studied for the effects of nonspecific nitrogen replacement of milk protein

Exp.	Subject	Age	Height	Body wt		Calorie intake [1]
				Beginning	End	
		year	*cm*	*kg*	*kg*	*kcal/kg body wt*
1	WD	21	183	69.4	68.8	47
	WI	19	165	66.6	64.4	52
	DK	23	183	67.1	67.7	47
	CK	23	170	60.0	59.5	44
	RR	19	180	74.6	70.5	42
	LD	21	173	77.7	77.1	40
	RB	20	173	85.4	85.0	34
2	PD	19	181	72.1	71.2	51
	KE	24	180	76.4	76.9	44
	ND	19	162	61.3	62.6	42
	TJ	27	175	68.0	69.4	49
	JR	22	183	75.1	75.7	45
	MS	18	177	71.4	72.6	45
	RW	23	179	74.1	74.0	49
3	KE	24	180	82.4	83.5	42
	DH	20	167	84.2	84.1	42
	JL	19	181	78.5	78.9	42
	SM	22	163	71.0	71.2	45
	JR	22	183	77.7	78.4	44
	ER	19	177	77.7	77.4	46
	GS	23	185	88.1	88.8	50
	IS	20	187	65.6	67.5	48
	LD	22	173	77.1	75.9	42
	WI	19	165	69.1	67.5	50

[1] Calculated from food composition tables (Watt, B. K., and A. L. Merrill 1963 Composition of Foods. Agriculture Handbook 8, Washington, D.C.) and data supplied by the manufacturers of some of the dietary components.

from 14 to 18 days, depending upon the time required for the subject to reach an apparently steady state of nitrogen metabolism as revealed by urinary nitrogen excretion.

TABLE 2

Composition of diet given to young men studied for the effects of replacement of milk protein with nonspecific nitrogen

Ingredient	Amount per day [1,2]
	g
Nonfat dried skim milk [3]	65.6
Oatmeal [4]	110.8
Dexin [5]	130.0
Oil [6]	140.0
Vanilla	10.0
Lemon juice	5.0
NaCl	1.0
Avicel [7]	5.0
K_2HPO_4	3.5
H_2O	500.0

[1] Amount for a 70-kg subject. Intake for each subject adjusted according to body weight.

[2] In addition, cornstarch cookies and Danish dessert (cornstarch, sucrose and artificial flavoring and coloring), and carbonated beverages were given according to individual caloric needs. Supplemental iron (1 tablet supplying 14 mg Fe, kindly supplied by Smith, Kline & French, Inc., Philadelphia) and a Unicap (Upjohn Company, Kalamazoo, Mich.) multivitamin capsule were given daily to all subjects. Each capsule is stated to contain: (in milligrams) vitamin A, 1.5; vitamin D, 0.012; vitamin B_1·HCl, 2.5; vitamin B_2, 2.5; vitamin C, 50; nicotinamide, 20; Ca pantothenate, 5; pyridoxine·HCl, 0.5; and cobalamin, 0.002. Subjects were also allowed Trident Gum (donated by American Chicle Company, New York). Five or six salt tablets, each supplying 1 g NaCl, were given to provide additional sodium required.

[3] Carnation Company, Van Nuys, Calif.

[4] Dried, cooked Buckeye Homogenized Rolled Oats, kindly supplied by Quaker Oats Company, Barrington, Ill.

[5] Burroughs Wellcome and Co., Tuckahoe, N. Y. Approximate composition: dextrins 75%; maltose 24%.

[6] Corn oil, Mazola, Best Foods Division, Corn Products Company, New York.

[7] F.M.C. Corporation, Marcus Hook, Penna. Microcrystalline alpha cellulose.

TABLE 3

Composition of mixture of nonessential amino acids used to dilute milk protein

Amino acid	By wt
	%
L-Arginine·HCl	6.15
L-Histidine·HCl, H_2O	4.44
L-Alanine	5.80
L-Aspartic acid	12.28
L-Glutamic acid	39.26
Glycine	3.33
L-Proline	18.72
L-Serine	9.93
Total	100

Three experiments were conducted. The first two, with seven subjects each, evaluated the effects of replacing 20 and 25%, respectively, of dietary protein by a nonspecific nitrogen source, a mixture of glycine and diammonium citrate. Experiment 3, with 10 subjects, explored the effects of 25% isonitrogenous replacement of milk protein by a mixture of nonessential amino acids (table 3). The dilution period lasted for 20 or 21 days after which the subjects were returned to the basal diet for a period of 7 to 10 days. After basal period 2 the experiment was terminated.

Handling and analyses of samples. Urine was collected for complete 24-hour periods. Total dietary nitrogen, urinary nitrogen and creatinine were analyzed as described previously (9, 10). Urea nitrogen (10) and total sulfur (18) were measured on pooled urine samples and serum total protein determined (19). On the final day of each dietary period a blood sample was taken at 8AM after an overnight fast (9 to 10 hours), and plasma free amino acids were measured by ion exchange chromatography.[5] The plasma was prepared for analysis as described by Mechanic et al. (20). Tryptophan was determined according to the procedure of Duggan and Udenfriend (21). Results for urinary nitrogen were evaluated by the *t* test and by the paired *t* test for plasma amino acids.

RESULTS

Total urinary nitrogen excretion during the experimental periods of both the 20 and 25% replacement of milk protein by glycine and diammonium citrate is given in table 4. For the 20% replacement (exp. 1) no significant increase in nitrogen excretion was observed in any of the seven subjects at this dilution when compared with the initial basal period. Three subjects, however, showed a significantly lower urinary nitrogen excretion during the final basal period than during the initial basal period.

During the 25% dilution period (exp. 2), three of the seven subjects showed a significantly higher urinary nitrogen excretion compared with basal period 1, and most of the subjects showed a decreased

[5] Technicon Amino Acid Analyzer, Technicon Corporation, Ardsley, N. Y.

rate of urinary nitrogen excretion when they were returned to the basal diet at the end of the experiment (table 4). The increased nitrogen retention during this final period, as compared with the dilution period, was statistically significant ($P < 0.01$) in four of the seven subjects (table 4). The results suggested that the nitrogen of the diet diluted 20% with glycine and diammonium citrate was not as well utilized as that of the 20% diluted and basal diets. The decreased urinary nitrogen excretion during basal period 2 of the experiment compared with basal period 1 suggests that dilution of the milk protein may have resulted in a depletion of the body's pool of an essential amino acid(s) during the period of replacement by nonspecific nitrogen.

As summarized in table 5, urea excretion followed the same trends as total urinary nitrogen excretion during the experimental periods of experiments 1 and 2. Subjects fed a 25% dilution diet showed a higher rate of urea nitrogen excretion during the dilution period, and most of them showed a lower urea nitrogen excretion upon return to the undiluted diet. The results for total urinary sulfur excretion and the total urinary N/S ratio are also given in table 5. Total sulfur excretion was decreased by about 10% during the 25% dilution period compared with basal period 1, and for some subjects sulfur excretion tended to return to initial levels during the final period on the basal diet.

Subject DK showed a substantially higher total urinary sulfur excretion during the 20% dilution period (table 5), and a markedly decreased total sulfur excretion when returned to the undiluted diet despite opposite trends in total S-amino acid intake. This subject also showed a significant increase in nitrogen retention when returned to the basal diet during the final period of the study. There may have been an interaction between the nonspecific nitrogen source and the utilization of S-amino acids in this individual. Moreover, three of the four subjects in experiment 2 (PD, KE and MS) showing lower total urinary nitrogen output during basal period 2 also showed a decrease in total urinary sulfur output. These results suggest that above a certain level which varies with individuals, the dilution of milk protein with glycine and diammonium citrate as a nonspecific nitrogen source influences the utilization and metabolism of the sulfur amino acids which are limiting in this protein (1).

TABLE 4

Effect of dilution of milk protein with glycine and diammonium citrate on urinary nitrogen excretion (g/day) by young men (exps. 1 and 2)

Subject	Nitrogen intake	Dilution of dietary protein			
		0%	20%	25%	0%
	g/day				
WD	4.22	3.92 ± 0.65(6) [1]	4.09 ± 0.86(13)	—	4.14 ± 1.08(7)
WI	4.08	4.61 ± 0.38(5)	4.08 ± 0.52(11) [a] *	—	3.70 ± 0.44(7) [a] **
DK	4.09	4.25 ± 0.54(9)	4.14 ± 0.39(11)	—	3.59 ± 0.36(7) [b] ** [a] *
CK	3.36	2.69 ± 0.67(6)	2.89 ± 0.32(8)	—	2.98 ± 0.42(6)
RR	4.56	3.76 ± 0.44(7)	4.26 ± 0.60(9)	—	3.89 ± 0.52(6)
LD	4.80	5.36 ± 0.33(8)	5.50 ± 0.23(9)	—	5.09 ± 0.67(9)
RB	4.91	4.43 ± 0.44(7)	4.48 ± 0.48(8)	—	3.74 ± 0.38(9) [b] ** [a] **
PD	4.39	4.11 ± 0.16(5)	—	4.67 ± 0.30(9) [a] **	4.02 ± 0.32(10) [b] **
KE	4.65	4.42 ± 0.33(8)	—	4.51 ± 0.22(10)	3.95 ± 0.47(10) [b] ** [a] *
ND	3.73	3.14 ± 0.27(6)	—	3.52 ± 0.52(10)	3.19 ± 0.34(9)
TJ	4.14	3.49 ± 0.22(5)	—	3.67 ± 0.30(6)	3.37 ± 0.24(10)
JR	4.57	4.12 ± 0.17(5)	—	4.66 ± 0.33(9) [a] **	4.16 ± 0.37(9) [b] **
MS	4.35	3.44 ± 0.30(6)	—	4.27 ± 0.33(11) [a] **	3.52 ± 0.28(9) [b] **
RW	4.51	5.07 ± 0.50(7)	—	4.88 ± 0.34(11)	4.65 ± 0.60(10)

[1] Mean ± SD for number of observations shown in parentheses. Total lengths of dietary periods were 14 to 16, 20 and 7 to 10 days for the 0%, 20 or 25% and 0% dilution periods, respectively.
[a] Significantly different from basal period 1.
[b] Significantly different from dilution period.
* $P < 0.05$.
** $P < 0.01$.

TABLE 5

Effect of dilution of milk protein with nonspecific nitrogen on urinary urea, sulfur (g/day) and the total N/S ratio in young men [1]

Exp. no.	Subject	Urea nitrogen	Sulfur	N/S [2]	Urea nitrogen	Sulfur	N/S	Urea nitrogen	Sulfur	N/S
1					Dilution of protein by glycine and diammonium citrate					
			0%			20%			0%	
	WD	2.3	0.255	15.4	2.9	0.292	14.0	2.2	—	—
	WI	3.1	0.351	13.1	2.4	0.285	14.3	2.2	0.351	10.5
	DK	2.7	0.351	12.1	2.3	0.516	8.0	1.4	0.123	29.2
	Mean	2.7	0.319	13.5	2.5	0.364	12.1	1.9	0.237	19.9
2			0%			25%			0%	
	PD	2.8	0.357	11.5	3.1	0.324	14.4	2.2	0.264	15.2
	KE	2.9	0.348	12.7	3.0	0.318	14.2	2.3	0.281	12.8
	ND	2.2	0.224	14.0	2.4	0.198	17.8	1.9	0.216	14.8
	TJ	2.7	0.324	10.8	2.8	0.246	14.9	2.0	0.240	14.0
	JR	2.9	0.333	12.4	3.5	0.273	17.1	2.5	0.297	14.0
	MS	1.9	0.252	13.6	2.4	0.252	16.9	2.2	0.221	15.9
	RW	3.0	0.369	13.7	3.5	0.342	14.3	3.5	0.363	12.8
	Mean	2.6	0.315	12.7	3.0	0.279	15.7	2.4	0.260	14.2
	SD	0.4	0.055	1.2	0.5	0.051	1.5	0.5	0.050	1.2
3					Dilution of protein by nonessential amino acids					
			0%			25%			0%	
	KE	2.6	0.327	13.1	3.0	0.285	15.6	2.6	0.321	11.9
	DH	3.6	0.423	12.3	2.9	0.342	12.8	3.6	0.384	11.5
	JL	3.0	0.393	11.2	2.9	0.282	15.2	2.7	0.339	11.8
	SM	2.5	0.282	13.9	2.6	0.216	17.3	2.6	0.276	13.9
	JR	2.7	0.303	14.0	3.0	0.306	13.7	2.6	0.297	12.8
	ER	2.5	0.303	12.4	3.0	0.261	15.9	3.1	0.297	14.1
	GS	2.4	0.324	14.9	2.9	0.273	16.3	2.1	0.318	11.6
	IS	1.5	0.294	9.4	3.2	0.336	12.8	3.5	0.318	14.3
	LD	3.2	0.411	11.4	3.3	0.363	12.6	3.3	0.375	12.3
	WI	2.0	0.341	11.8	2.3	0.285	11.5	2.5	0.341	11.3
	Mean	2.6	0.339	12.4	2.9	0.295	14.4	2.9	0.326	12.5
	SD	0.6	0.050	1.6	0.3	0.042	1.9	0.5	0.033	1.1

[1] Analysis on pooled urine samples for periods shown in tables 4 and 6.
[2] Ratio of total urinary nitrogen to total urinary sulfur. Values for total nitrogen taken from tables 4 and 6.

The results given in tables 4 and 5 show that total nitrogen and urea nitrogen output increased during a 25% dilution with glycine and diammonium citrate and decreased to a variable degree when subjects were returned to the basal diet. The question remained as to whether the glycine and diammonium citrate mixture was the most effective source of nonspecific nitrogen. Accordingly, a mixture of nonessential amino acids (table 3) was tested at the 25% level of dietary protein dilution. In this experiment (table 6) only 2 of the 10 subjects showed a statistically significant decrease in urinary nitrogen output following return to the basal diet after the 3-week period of 25% dilution with the NEAA mixture. Three subjects showed a lower urinary nitrogen excretion during basal period 2 as compared with the initial basal period. Subject KE previously showed a similar response following a 25% dilution of the dietary nitrogen with glycine and diammonium citrate (table 4).

Urinary nitrogen output during the dilution period did not increase significantly above that of the initial basal period, except in the case of two subjects. In one case (IS), however, a relatively low rate of excretion was observed during basal period 1, and during the final basal period urinary nitrogen output was the same as that during the dilution period. The low initial basal level of nitrogen excretion was probably atypical but the reason is unknown.

The results for urea nitrogen and total urinary sulfur excretion for the 10 subjects given nonessential amino acids as the source of nonspecific nitrogen are sum-

TABLE 6

Effects of dilution of milk protein with a mixture of nonessential amino acids on urinary nitrogen excretion by young men (exp. 3)

Subject	Nitrogen intake	Dilution of dietary protein		
		0%	25%	0%
	g/day	*g N/day*	*g N/day*	*g N/day*
KE	5.01	4.30 ± 0.20(8) [1]	4.46 ± 0.21(8)	3.84 ± 0.32(9) [a] ** [b] **
DH	5.12	5.22 ± 0.58(8)	4.38 ± 0.43(5) [a] *	4.40 ± 0.44(7) [a] **
JL	4.77	4.42 ± 0.33(7)	4.28 ± 0.33(9)	4.01 ± 0.69(9)
SM	4.32	3.93 ± 0.36(10)	3.74 ± 0.38(10)	3.85 ± 0.60(9)
JR	4.73	4.25 ± 0.20(6)	4.19 ± 0.33(9)	3.80 ± 0.31(7) [a] ** [b] *
ER	4.73	3.77 ± 0.96(10)	4.14 ± 0.46(9)	4.19 ± 0.57(9)
GS	5.38	4.38 ± 0.67(12)	4.45 ± 0.80(9)	3.70 ± 0.73(9)
IS	3.99	2.78 ± 0.10(8)	4.29 ± 0.55(9) [a] **	4.55 ± 0.45(9) [a] **
LD	4.77	4.70 ± 0.45(6)	4.56 ± 0.66(8)	4.61 ± 0.65(8)
WI	4.16	4.04 ± 0.62(5)	3.27 ± 0.47(6)	3.85 ± 0.44(7)

[1] Mean ± SD for number of observations shown in parentheses. Total length of 0, 25 and 0% dietary periods were 16 to 18, 20 to 21 and 9 days, respectively.
[a] Significantly different from basal period 1.
[b] Significantly different from dilution period.
* $P < 0.05$.
** $P < 0.01$.

marized in table 5. During the dilution period, total sulfur excretion was lower than during the initial basal period. The mean decrease in total urinary sulfur excretion was slightly greater during this period than in the corresponding period of experiment 2 involving use of glycine and diammonium citrate.

In contrast to results obtained in experiment 2 (table 5), total urinary sulfur excretion did not appear to decrease further during basal period 2 for KE, DH and JR. These three subjects showed increased nitrogen retention in the final basal period as compared with the initial basal period. In experiment 2 with glycine and diammonium citrate, a lower sulfur output was observed during basal period 2 for the three subjects (PD, KE and MS) who showed increased nitrogen retention after the 20-day period of dilution. These results suggest that the mixture of NEAA may be a slightly more effective source of nonspecific nitrogen for the isonitrogenous replacement of skim milk protein than glycine and diammonium citrate.

Serum total protein and the concentrations of free amino acids determined for subjects studied at a 25% level of replacement with the two sources of nonspecific nitrogen are summarized in table 7. No significant differences ($P > 0.05$) in the concentrations of these blood constituents were detected. During dilution with nonessential amino acids (exp. 3) only alanine increased, but because of high variability this change was not statistically significant.

DISCUSSION

The results of urinary nitrogen excretion in the present experiments indicate that for most human subjects, milk protein, when fed at about the minimum requirement level (0.38 g protein/kg per day), can be replaced with 20% of nitrogen from nonspecific nitrogen sources without affecting its nutritive value. The extent of replacement possible varied with the subjects from about 20% to at least 25%; this may reflect variability in the requirement for individual essential amino acids (5, 6). The influence of dilution appeared to be partially affected by the source of nitrogen used for the replacement of milk protein nitrogen, because glycine and diammonium citrate resulted in a greater increase in urinary nitrogen excretion during the 25% dilution period than the isonitrogenous mixture of nonessential amino acids.

Kofranyi and Jekat (22) in their study with adult subjects concluded that only 10 to 15% of milk protein could be replaced with ammonium citrate without a change in the value of the protein. The difference between their results and ours may be due to their limited number of subjects and use of ammonium citrate alone as the nonspecific nitrogen source. Clark et al.

TABLE 7

Serum total protein and free amino acids in fasting plasma of young men studied for effects of dilution of milk protein with nonspecific nitrogen

Amino acid	Dilution of protein					
	0%	25%	0%	0%	25%	0%
	Nonessential amino acids			Glycine and diammonium citrate		
Essential						
Threonine	186 ± 24 [1]	166 ± 42	174 ± 33	204 ± 31	211 ± 26	181 ± 26
Valine	198 ± 25	219 ± 64	210 ± 26	205 ± 29	174 ± 32	182 ± 40
Isoleucine	76 ± 14	88 ± 27	77 ± 7	86 ± 12	65 ± 6	63 ± 12
Leucine	124 ± 15	146 ± 44	134 ± 18	134 ± 25	115 ± 20	112 ± 23
Tyrosine	62 ± 9	68 ± 24	71 ± 10	77 ± 22	57 ± 11	55 ± 13
Phenylalanine	63 ± 7	67 ± 19	62 ± 5	64 ± 14	55 ± 8	55 ± 11
Lysine	187 ± 36	222 ± 75	201 ± 27	198 ± 28	164 ± 24	179 ± 29
Tryptophan	65 ± 8	72 ± 12	74 ± 9	61 ± 7	57 ± 10	63 ± 12
Nonessential						
Aspartic	67 ± 13	65 ± 15	52 ± 13	70 ± 10	70 ± 8	64 ± 7
Serine and glutamine	180 ± 52	232 ± 119	189 ± 66	149 ± 68	188 ± 22	169 ± 20
Glutamic	313 ± 38	334 ± 37	312 ± 73	341 ± 50	309 ± 55	289 ± 40
Glycine	307 ± 59	308 ± 123	294 ± 25	335 ± 25	401 ± 51	312 ± 56
Alanine	666 ± 136	809 ± 289	643 ± 188	557 ± 115	490 ± 127	501 ± 118
Histidine	120 ± 10	122 ± 30	117 ± 26	117 ± 21	92 ± 12	100 ± 4
Arginine	99 ± 32	130 ± 24	106 ± 20	121 ± 47	91 ± 19	74 ± 8
Serum protein (g/100 ml)	7.1 ± 0.4	7.2 ± 0.2	7.3 ± 0.4	7.0 ± 0.3	6.9 ± 0.3	7.0 ± 0.3

[1] Micromoles per liter. Mean ± SD for samples taken at 8:00 AM after a 9 to 10 hour fast at the end of each period.

(13) observed in young adults given 9.0 g nitrogen/day (of which approximately half was provided by the supplementary nonspecific nitrogen) that nitrogen retention was depressed when diammonium citrate was fed, but not when glycine alone was given. Although none of the seven young adults in the present study appeared to retain less nitrogen when 20% of milk protein was replaced with the glycine and diammonium citrate mixture, three appeared to show additional retention when returned to the basal diet. This latter observation may mean that an amino acid depletion had occurred which was not apparent from measuring urinary nitrogen excretion alone during the dilution period. This possibility was not reflected, however, by a reduction in the concentration of essential amino acids in fasting plasma samples. The results suggested that milk protein can be replaced to a greater extent in most young adults than indicated by the study of Kofranyi and Jekat (22).

Snyderman et al. (2) studied infants from 3 weeks to 5 months of age and found that weight gain and nitrogen balance could be maintained despite a partial substitution of evaporated cow's milk protein by glycine or urea. Although their experiments were not designed to determine the extent to which milk protein nitrogen could be replaced by nonessential nitrogen, it appears from their data that 20% of cow's milk protein could be isonitrogenously replaced by either glycine or urea without reducing infant growth. This is probably an overestimate, however, since the level of protein intake in their study was above minimum requirements for growth (23).

In establishing amino acid requirements of man, investigators have used different sources of supplemental nitrogen (11–14). Swendseid et al. (24) have reported that glycine alone is not well used by man as a nonspecific nitrogen source; however, they observed that a combination of glycine and diammonium citrate was as effective a source of nonspecific nitrogen as a mixture of nonessential amino acids. Experiments with weanling rats have shown that different sources of nonspecific nitrogen can affect growth rate in the presence of minimal amounts of essential amino acids (25, 26). Swendseid et al. (27) have

shown that the growth rate of rats may be depressed by a low protein diet supplemented with 7.5% glycine. The results of our study with young men suggest that there may be some difference in the utilization of glycine and diammonium citrate, compared with a mixture of nonessential amino acids, when included as nonspecific nitrogen supplements in a diet supplying nitrogen at approximately the minimum requirement.

In experimental animals glycine alleviates the growth-depressing effect of a high methionine intake (28, 29) and conversely, methionine protects against excess glycine (30). Sulfur amino acids are limiting in cow's milk protein and therefore, under conditions of a relatively low intake of these amino acids in the present studies, a comparison of the effects of glycine-diammonium citrate and a mixture of nonessential amino acids on urinary nitrogen excretion was considered appropriate. Although a close relationship between urinary nitrogen and sulfur excretion has been noted by a number of investigators (31, 32), a difference in the pattern of excretion between these elements has also been reported (33). Our results with subjects who reacted to a 20 or 25% dilution with glycine-diammonium citrate by showing changes in urinary sulfur excretion suggest that this nitrogen source may affect S-amino acid metabolism at the level of protein intake used in this study. Further studies are required to evaluate more fully the significance of these observations.

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LITERATURE CITED

1. Food and Agriculture Organization 1965 Protein Requirements Report of a Joint FAO/WHO Expert Group. FAO Nutr. Meetings, Rome, Italy, Rep. Ser., 37, p. 39.
2. Snyderman, S. E., L. E. Holt, Jr., J. Dancis, E. Roitman, A. Boyer and M. E. Balis 1962 "Unessential nitrogen." A limiting factor for human growth. J. Nutr., *78:* 57.
3. Kies, C., E. Williams and H. M. Fox 1965 Effect of "non-specific" nitrogen intake on adequacy of cereal proteins for nitrogen retention in human adults. J. Nutr., *86:* 357.
4. Kies, C., H. M. Fox and E. R. Williams 1967 Time, stress, quality and quantity as factors in the nonspecific nitrogen supplementation of corn protein for adult men. J. Nutr., *93:* 377.
5. Rose, W. C. 1957 The amino acid requirements of adult man. Nutr. Abstr. Rev., *27:* 631.
6. Hegsted, D. M. 1963 Variation in requirements of nutrients—amino acids. Federation Proc., *22:* 1424.
7. Rose, W. C., M. J. Oesterling and M. Womack 1948 Comparative growth on diets containing ten and nineteen amino acids with further observations upon the role of glutamic and aspartic acids. J. Biol. Chem., *176:* 753.
8. Young, V. R., and J. Zamora 1968 Effects of altering the proportions of essential to nonessential amino acids on growth and plasma amino acid levels in the rat. J. Nutr., *96:* 21.
9. Scrimshaw, N. S., V. R. Young, R. Schwartz, M. L. Piché and J. B. Das 1966 Minimum dietary essential amino acid-to-total nitrogen ratio for whole egg protein fed to young men. J. Nutr., *89:* 9.
10. Huang, P. C., V. R. Young, B. Cholakos and N. S. Scrimshaw 1966 Determination of the minimum dietary essential amino acid-to-total nitrogen ratio for beef protein fed to young men. J. Nutr., *90:* 416.
11. Rose, W. C., and R. L. Wixom 1955 The amino acid requirements of man. XVI. The role of the nitrogen intake. J. Biol. Chem., *217:* 997.
12. Leverton, R. M., M. R. Gram, M. Chaloupka, E. Brodovsky and A. Mitchell 1956 The quantitative amino acid requirements of young women. I. Threonine. J. Nutr., *58:* 59.
13. Clark, H. E., N. J. Yess, E. J. Vermillion, A. F. Goodwin and E. T. Mertz 1963 Effect of certain factors on nitrogen retention and lysine requirements of adult human subjects. III. Source of supplementary nitrogen. J. Nutr., *79:* 131.
14. Holt, L. E., Jr., and S. E. Snyderman 1965 Protein and amino acid requirement of infants and children. Nutr. Abstr. Rev., *35:* 1.
15. Watts, J. H., L. Bradley and A. N. Mann 1965 Total N, urea, and ammonia excretions of human male subjects fed several nonessential amino acids singly as the chief source of nonspecific N. Metabolism, *14:* 504.
16. Bender, A. E., and B. H. Doell 1957 Note on the determination of net protein utilization by carcass analysis. Brit. J. Nutr., *11:* 138.
17. Orr, M. L., and B. K. Watt 1957 Amino acid content of foods. Home Econ. Res. Rep. no. 4, USDA, Washington, D. C.
18. Peters, J. P., and D. D. Van Slyke 1946 Quantitative Clinical Chemistry, vol. 2. Williams & Wilkins Company, Baltimore, p. 890.

19. Annino, J. S. 1964 Clinical Chemistry. Little, Brown and Company, Boston, p. 184.
20. Mechanic, G., M. L. Efron and V. E. Shih 1966 A rapid quantitative estimation of tyrosine and phenylalanine by ion-exchange chromatography. Anal. Biochem., *16:* 420.
21. Duggan, D. E., and S. Udenfriend 1956 The spectrophotofluorometric determination of tryptophan in plasma and of tryptophan and tyrosine in protein hydrolysates. J. Biol. Chem., *223:* 313.
22. Kofranyi, E., and F. Jekat 1964 Zur Bestimmung der biologischen Wertigkeit von Nahrungsproteinen. IX. Der Ersatz von hochwertigem Eiweiss durch nichtessentiellen Stickstoff. Hoppe-Selyer's Z. Physiol. Chem., *338:* 154.
23. Chan, H., and J. C. Waterlow 1966 The protein requirement of infants at the age of about 1 year. Brit. J. Nutr., *20:* 775.
24. Swendseid, M. E., C. L. Harris and S. G. Tuttle 1960 The effect of sources of nonessential nitrogen on nitrogen balance in young adults. J. Nutr., *71:* 105.
25. Sauberlich, H. E. 1961 Studies on the toxicity and antagonism of amino acids for weanling rats. J. Nutr., *75:* 61.
26. Swendseid, M. E., J. B. Hickson and B. W. Friedrich 1962 Effect of nonessential nitrogen supplements on growth and on the amino acid content in plasma and muscle of weanling rats fed a low-protein diet. J. Nutr., *78:* 115.
27. Swendseid, M. E., J. Villalobos and B. Friedrich 1963 Ratios of essential-to-nonessential amino acids in plasma from rats fed different kinds and amounts of proteins and amino acids. J. Nutr., *80:* 99.
28. Roth, J. S., and J. B. Allison 1949 The effect of feeding excess glycine, L-arginine and DL-methionine to rats on a casein diet. Proc. Soc. Exp. Biol. Med., *70:* 327.
29. Benevenga, N. J., and A. E. Harper 1967 Alleviation of methionine and homocystine toxicity in the rat. J. Nutr., *93:* 44.
30. Wertz, J. M., C. R. Seward, E. L. Hove and J. S. Adkins 1968 Interrelationship of dietary glycine, methionine and vitamin E in the rat. J. Nutr., *94:* 129.
31. Eckert, J. N. 1948 Sulfur balance indexes of casein in adult dogs with and without addition of DL-methonine. Arch. Biochem. Biophys., *19:* 379.
32. Wright, J. B., P. G. Martin, M. L. Skellenger and D. S. Moschette 1960 Metabolic patterns in pre-adolescent children. III. Sulfur balance on three levels of nitrogen intake. J. Nutr., *72:* 314.
33. Bressani, R., J. E. Braham, L. E. Elias and R. Balconi 1965 Urinary nitrogen and sulfur excretion in dogs under different dietary treatments. J. Nutr., *87:* 77.

Neuromotor Development in Progeny of Underfed Mother Rats [1]

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ABSTRACT The neuromotor development of progeny from mothers subjected to 50% dietary restriction during either 1) gestation and lactation or 2) gestation alone, and of appropriate control progeny was observed twice each week from week 1 to week 7 of life. The ages at which several coordinated movements, behavior patterns and reflex responses first appeared were recorded for experimental progeny and their controls. The longer period of maternal dietary restriction resulted in developmental delays of as much as 2 weeks, and lesser but consistent delays occurred after the more limited restriction. In addition, several clearly abnormal types of activity occurred in both types of experimental progeny, but never at any time in the controls. The delays and abnormalities in the progeny of mothers restricted during gestation alone were not accompanied by corresponding deficits in weight during the period of observation. The results demonstrate that these aspects of neuromotor integration do not necessarily bear a close relationship to chronological age.

The nutrition of the mother during reproduction in rats is now known to have important influence on the physical and metabolic development of the offspring (1–6). When the dietary intake of female rats is reduced to one-half that consumed by control animals fed ad libitum during gestation and lactation the offspring are permanently stunted in growth and their metabolism is abnormal, despite their receiving a fully adequate diet after weaning (1, 2). Comparable effects occur when the utilizable protein in the maternal diet is reduced (3–6). If the stress to the offspring is confined to the period of intrauterine development by fostering the newborn pups to normally fed mothers the effects are more subtle but nonetheless important.[2] In such circumstances there is little or no difference in body weight between experimental and control animals during the period of rapid growth; only as a plateau is approached does the growth of the experimental progeny begin to lag. This observation emphasizes the importance of following the offspring at least until maturity before excluding the presence of abnormalities resulting from perinatal malnourishment.

It was natural to inquire whether the physical and metabolic abnormalities already demonstrated are also accompanied by neurological and behavioral abnormalities. It is true that the brain enjoys some degree of differential protection from adverse nutritional conditions — a phenomenon often described as "brain sparing." It is also true that the fetus as a whole is to some extent protected from the full impact of maternal malnourishment by mechanisms which are not yet well understood but which have been popularized by the phrase, "fetal parasitism." From frequent repetition these shibboleths have acquired connotations well beyond those justified by the data which led to their formulation. In particular the partial and quantitative protections which they describe have been interpreted as complete and qualitative. Both these principles in fact describe no more than a relative preservation of tissue mass. The available data do not permit the inference that functional integrity and developmental potential are unimpaired. That there was unwarranted confidence in the welfare of the fetus during maternal nutritional stress is

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[2] Stephan, J. K., and B. F. Chow 1968 Growth of progeny from rats underfed during gestation only. Federation Proc., 27: 728 (abstract).

now apparent, and there are already data from animals to suggest that the brain does not escape damage under such conditions (6, 7).

The specific objectives of the experiments now reported were to compare with corresponding control animals the ages at which certain neuromotor capabilities appeared in a) progeny of mothers subjected to overall dietary restriction throughout gestation and lactation, and b) progeny which were born to mothers subjected to dietary restriction during gestation but which were fostered to normally fed mothers on the day following birth.

EXPERIMENTAL PROCEDURES AND METHODS

The sires and dams of the experimental and control animals were drawn from the colony of McCollum rats maintained in this laboratory. The same standard commercial diet [3] which is used routinely in the colony was also used throughout these experiments. The procedure for mating has been described previously (3). Immediately after sperm were seen in a vaginal lavage each female was individually housed and randomly assigned to the experimental or control group. Animals in the control group continued to be fed ad libitum; experimental animals received 10 g of feed daily throughout gestation, an approximate 50% restriction. After delivery the procedure differed in each of the three experiments, as described below.

Experiment 1. On the day following birth, litters containing more than eight pups were reduced to that number and any litters containing fewer than six pups were excluded from the experiment. The daily ration of the experimental mothers was increased to 20 g which represents an approximate 50% restriction for the period of lactation. All mothers were fed ad libitum after day 21 of lactation, but the pups were left with the mother until day 28 because of a previously observed delay in opening of the eyes among such experimental progeny. Experimental and control pups which have been prepared in this way are designated RR and NN, respectively. Eight RR and eight NN pups, each randomly selected from different litters on day 5 after birth, were used in this experiment. Two RR animals died within the first 2 weeks of the study; observations obtained earlier on these two animals were excluded.

Experiment 2a. On the day following delivery all pups from both groups were removed from their own mothers. The unrestricted mothers were used as foster mothers, each being assigned eight pups, four from each of two mothers. Experimental and control pups were not mixed within litters and no pup was nursed by its own mother. The restricted females and the surplus pups were not used further in the experiment. The foster mothers all continued to be fed ad libitum. Weaning was deferred until day 28. The experimental pups are designated RN cf, indicating that the maternal diet was restricted during gestation and normal in lactation and that the pups were cross-fostered. The corresponding control animals are designated NN cf. Eight RN cf and eight NN cf pups, all born to different mothers, were randomly selected for observation in the development box. Two NN cf animals died — one at 14 days of age and one at 24 days of age; data obtained earlier on these two animals were excluded.

Experiment 2b. The procedure differed only in detail from that of experiment 2a. The offspring were randomly assigned to foster mothers on the day following birth but with the constraints that no pups were nursed by their own mother, and experimental animals were not placed in the same litter as controls. The foster litters were limited to eight pups. Eleven RN cf pups and 12 NN cf pups were randomly selected for observation. One RN cf animal died at 10 days of age; all data from this animal were excluded. A further RN cf animal ceased to gain weight at 30 days of age and died at 56 days of age, shortly after the end of the study.

After weaning on day 28 after birth, all pups in each of the three experiments were individually housed and offered the commercial diet ad libitum.

The technique of assessment used throughout all these experiments was derived from that of Frankova (8). The observations were made in an open-topped Lucite box with a floor measuring 30 cm

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

by 30 cm and sides 10-cm high. The floor was marked into nine 10-cm squares. On day 5 or 6 after birth, and twice each week thereafter until approximately 7 weeks after birth, each pup was placed in the center square and observed for a period of 6 minutes. The external environment was kept as uniform as possible by avoiding bright lights, draughts and noise. The box was cleaned thoroughly after each observation period to remove any olfactory traces. The first appearance of horizontal movement was arbitrarily defined as the first departure from the center square. The development of spontaneous activity was also assessed by the first appearance of head lifts, grooming, standing against the side of the box, standing alone, and finally escape from the box. The elicited responses, for which the appropriate stimuli were applied in each test period were the righting, orienting, and startle reflexes. The age at which the eyes opened and testes descended was recorded. In addition to these specific observations, detailed descriptions of any unusual activity were made.

RESULTS

Experiment 1. The growth of the RR and NN animals shown in table 1 was typical of that previously seen in this laboratory. The average number of days after birth at which the various developmental levels were achieved are presented together with the standard errors of the means in table 2. By each one of the chosen criteria the development of the experimental pups was conspicuously retarded in relation to the controls. The mean body weight at which these same levels of achievement were reached is shown in table 3. In addition to the delays in development, several anomalous manifestations, not seen at any stage in the development of the controls, occurred in the RR animals; four such patterns were distinguished: 1) sustained purposeless locomotion over a small circular course ("circling"); 2) fine, repetitive and rhythmical movements of the head and trunk with a periodicity of 2 to 5/second ("tremor"); 3) twitching of the limbs,

TABLE 1

Mean body weights of six male perinatally malnourished (RR) and eight male control (NN) progeny during the period of observation (exp. 1)

Age	Mean body wt	
	RR	NN
days	*g*	*g*
5	10.4(0.7) [1]	12.6(0.5) [1]
12	12.7(1.1)	23.9(0.9)
19	13.7(1.3)	39.7(1.1)
26	20.1(2.6)	69.6(1.2)
33	49.1(5.9)	106.9(4.6)
40	65.4(5.8)	137.4(3.8)
47	92.7(7.2)	170.7(4.6)

[1] SE of the mean.

TABLE 2

Mean age of appearance of developmental indices in six male perinatally malnourished (RR) and eight male control (NN) progeny (exp. 1)

Index	Mean age	
	RR	NN
	days	*days*
Horizontal movement	9.0(0.7) [1]	6.1(0.6) [1]
Vocalization	13.5(0.6)	6.5(0.6)
Righting	17.0(0.8)	6.5(0.6)
Head lift	13.9(0.5)	8.0(0)
Grooming	18.0(1.4)	13.9(0.5)
Orienting reflex	16.3(0.8)	12.0(0)
Startle reflex	18.8(0.9)	12.0(0)
Standing with support	22.2(0.9)	12.0(0)
Standing alone	42.5(1.5)	17.5(0.7)
Escape from box	42.3(2.3)	26.0(0)
Eye opening	20.0(0.6)	14.3(0.5)
Descent of testes	38.0(0.9)	27.9(0.6)

[1] SE of the mean.

TABLE 3

Mean body weights at which developmental indices appeared in six male perinatally malnourished (RR) and eight male control (NN) progeny (exp. 1)

Index	Mean body wt	
	RR	NN
	g	*g*
Horizontal movement	11.8(1.1) [1]	14.4(0.9) [1]
Vocalization	12.9(1.1)	15.1(1.2)
Righting	13.6(1.3)	14.9(1.0)
Head lift	13.1(1.2)	17.4(0.7)
Grooming	13.7(1.0)	28.5(1.3)
Orienting reflex	13.4(1.2)	23.9(0.9)
Startle reflex	13.9(1.1)	23.9(0.9)
Standing with support	16.0(1.6)	23.9(0.9)
Standing alone	75.4(8.3)	36.0(1.6)
Escape from box	74.8(2.7)	69.6(1.2)
Eye opening	14.7(1.7)	28.9(1.1)
Descent of testes	61.2(6.6)	78.1(2.9)

[1] SE of the mean.

followed by rigidity lasting at least 1 minute during which the animal was unresponsive to stimuli, and usually associated with defecation and micturition ("convulsion"); 4) adoption of a bizarre position maintained with complete immobility for at least 3 minutes during which time the animal was unresponsive to mild stimuli ("catatonic stance"). Circling and tremors were seen in each experimental animal at least once; convulsions were seen in seven of the eight and catatonic stances in four.

Experiment 2a. The mean weights of the RN cf and NN cf groups at various ages are shown in table 4. During the period covered by the study the two groups differed little in weight; this finding was consistent with that of other comparable experiments.[4] The days of age at which the specific neuromotor responses were observed or elicited appear in table 5. The differences between groups are smaller than those observed in experiment 1 and individually are only of marginal significance. The relative uniformity of the delays in this wide range of indexes, however, strongly suggest that maternal dietary restriction confined to gestation does indeed result in a degree of neuromotor retardation. These suggestive data are strengthened by the fact that the same types of abnormality which had been observed in the more severely stressed progeny of experiment 1 were again seen in this experiment, although less often. Circling occurred in all the experimental animals at least once; tremors were seen in four, convulsions in four and catatonic stances in one.

TABLE 4

Mean body weights of eight male congenitally malnourished (RN cf) and six male control (NN cf) progeny during the period of observation (exp. 2a)

Age	Mean body wt	
	RN cf	NN cf
days	*g*	*g*
9	15.8(1.3) [1]	18.8(0.47) [1]
16	27.6(2.3)	28.6(1.4)
23	42.1(3.7)	44.0(1.2)
30	68.5(5.1)	74.7(1.9)
37	111.1(8.4)	116.7(2.3)
44	144.5(9.1)	155.2(4.0)

[1] SE of the mean.

The mean body weights at which the various developmental levels were reached are shown in table 5.

Experiment 2b. This was essentially a replication of experiment 2a and was carried out to substantiate the tentative conclusions drawn from that experiment. The data for growth, neuromotor "milestones" and body weights at each level of development appear in tables 7, 8 and 9, respectively, which correspond to tables 4, 5 and 6 of experiment 2a. The pups in experiment 2b were not weighed until 9 days of age so the body weights at which horizontal movement, vocalization, righting reflex and head lifts first appeared were not available. Because a RN cf animal which died at 56 days of age had ceased to grow at 30 days of age, and was probably moribund from this time onward, data for the RN cf group after 30 days of age are presented both with and without this animal in tables 7, 8 and 9. The growth of both the control and experimental animals in this experiment was more rapid than the growth of the comparable animals in the previous experiment; this difference is probably attributable to seasonal variation which commonly has considerable effect on the growth of rats in this laboratory.

TABLE 5

Mean age of appearance of developmental indices in eight congenitally malnourished (RN cf) and six male control (NN cf) progeny (exp. 2a)

Index	Mean age	
	RN cf	NN cf
	days	*days*
Horizontal movement	7.5(0.6) [1]	6.0(0) [1]
Vocalization	6.0(0)	6.0(0)
Righting	9.8(1.0)	8.0(0.6)
Head lift	9.1(0.7)	7.0(0.6)
Grooming	15.4(0.8)	12.7(1.4)
Orienting reflex	13.8(0.2)	13.0(0)
Startle reflex	16.6(0.8)	13.0(0)
Standing with support	16.0(0)	11.0(0.9)
Standing alone	25.9(0.9)	21.5(0.7)
Escape from box	28.1(2.8)	25.7(1.9)
Eye opening	17.5(0.7)	14.5(0.7)
Descent of testes	31.5(0.9)	28.5(0.7)

[1] SE of the mean.

[4] See footnote 1.

TABLE 6

Mean body weights at which developmental indices appeared in eight male congenitally malnourished (RN cf) and six male control (NN cf) progency (exp. 2a)

Index	Mean body wt	
	RN cf	NN cf
	g	*g*
Horizontal movement	13.7(1.3) [1]	13.6(0.2) [1]
Vocalization	11.2(0.7)	13.6(0.2)
Righting	16.9(2.0)	17.4(1.2)
Head lift	15.9(1.8)	15.3(1.2)
Grooming	26.3(2.5)	24.8(2.5)
Orienting reflex	24.0(2.4)	25.2(1.3)
Startle reflex	29.0(3.1)	25.2(1.3)
Standing with support	27.6(2.3)	22.6(1.9)
Standing alone	53.1(6.3)	38.1(2.3)
Escape from box	60.5(5.2)	56.7(7.8)
Eye opening	30.1(2.9)	27.2(1.9)
Descent of testes	77.6(7.3)	67.9(2.2)

[1] SE of the mean.

TABLE 7

Mean body weights of 11 male congenitally malnourishd (RN cf) and 12 male control (NN cf) progeny during the period of observation (exp. 2b)

Age	Mean body wt	
	RN cf	NN cf
days	*g*	*g*
9	18.2(0.7) [1]	20.6(0.7) [1]
16	30.4(0.6)	32.3(1.5)
23	50.6(4.6)	53.7(2.8)
30	77.0(3.9) [79.5] [2]	81.8(3.5)
38	120.2(7.9) [125.9] [2]	130.7(5.0)
44	146.9(10.5)[155.8] [2]	160.0(5.6)

[1] SE of the mean.
[2] Mean of RN cf group excluding animal which ceased to grow at 30 days of age and died at 56 days of age.

DISCUSSION

The observations in experiment 1 show beyond reasonable doubt that maternal dietary restriction throughout gestation and lactation caused considerable retardation in neuromotor development in addition to the previously demonstrated stunting of physical growth. But it is important to bear in mind that these experimental animals were currently undernourished during the first half of the observation period, and had probably not recovered sufficiently to have obtained full nutritional equilibrium before the end of the experiment. It cannot be concluded from the observed delays that permanent impairment of neuromotor development had taken place. However, the subsequent finding of impaired maze performance in adult RR progeny as compared with NN progeny [5] suggests that permanent neurological damage does indeed result from maternal dietary restriction during gestation and lactation. Nor was it clear from experiment 1 that any of the effects were necessarily attributable to fetal as opposed to neonatal nutritional deficit. Lat and his co-workers (9) demonstrated reduced activity in rats which had been nursed in large litters of 15 to 20 compared with rats nursed in abnormally small litters of three. Although these workers did not record the actual age of onset of particular types of activity it is reasonable to suppose from the data which are given that standing and grooming did, in fact, appear later in the more slowly growing animals which had been nursed in large litters. But it cannot be assumed that the entire difference between the two types of animal represents *subnormality* of those from large litters; it is probable that the animals reared in small litters also differed from the expected behavior of animals reared in litters of physiological size. Moreover, neither in the experiments of Lat et al. nor in experiment 1 reported here can the observed differences in behavior be attributed entirely to differences in *nutrition* of the fetus or neonate. Extreme variations in litter size may well have direct influence on behavioral development over and above any nutritionally mediated effect. Other, but perhaps equally important nonnutritional influences may ensue from our own technique. Restricted mothers are not, in general, good mothers. They are preoccupied by the search for food and are highly irritable. It would be surprising if the behavioral development of the progeny was not to some extent influenced by this atypical conduct of the mother during lactation.

The experiments involving manipulation of litter size had shown that particular levels of activity were more closely associated with body weight than with chronological age. The data relating to the first appearance of behavioral patterns in the

[5] Simonson, M., R. W. Sherwin, H. H. Hanson, and B. F. Chow 1968 Maze performance in offspring of underfed mother rats. Federation Proc., 27: 727 (abstract).

TABLE 8

Mean age of appearance of developmental indices in 11 male congenitally malnourished (RN cf) and 12 male control (NN cf) progeny (exp. 2b)

Index	Mean age		
	RN cf		NN cf
	days		*days*
Horizontal movement	8.3(0.7) [1]		6.0(0) [1]
Vocalization	6.3(0.3)		6.0(0)
Righting	10.6(0.9)		6.3(0.3)
Head lift	13.3(0.3)		9.1(0.4)
Grooming	14.1(0.5)		11.3(0.6)
Orienting reflex	16.6(1.0)		13.3(0.3)
Startle reflex	18.4(0.9)		14.6(0.7)
Standing with support	17.0(0.7)		13.0(0)
Standing alone	23.1(0.8)		19.6(0.5)
Escape from box	28.7(1.0)	[28.2(0.9)] [2]	23.3(0.1)
Eye opening	17.8(0.6)		14.1(0.6)
Descent of testes	32.9(0.6)	[32.8(0.6)] [2]	27.8(0.4)

[1] SE of the mean.
[2] Mean and SE of the mean for RN cf group excluding animal which ceased to grow at 30 days of age and died at 56 days of age.

TABLE 9

Mean body weights at which developmental indices appeared in 11 male congenitally malnourished (RN cf) and 12 male control (NN cf) progeny (exp. 2b)

Index	Mean body wt		
	RN cf		NN cf
	g		*g*
Grooming	27.4(0.7) [1]		25.4(1.8) [1]
Orienting reflex	33.2(2.6)		28.2(1.4)
Startle reflex	36.9(1.9)		30.4(1.9)
Standing with support	32.1(1.2)		27.7(1.3)
Standing alone	49.8(2.1)		42.8(2.6)
Escape from box	72.0(4.5)	[73.3(4.8)] [2]	54.9(2.5)
Eye opening	34.8(2.1)		28.3(1.3)
Descent of testes	92.2(5.8)	[95.5(5.3)] [2]	72.9(3.4)

[1] SE of the mean.
[2] Mean and SE of the mean for RN cf group excluding animal which ceased to grow at 30 days of age and died at 56 days of age.

experiment [6] do not show the association with body weight to be any closer than that with chronological age. In general, particular stages of development were achieved at considerably lower body weights in the perinatally malnourished animals than in the controls. Standing alone was a notable exception to this general tendency and did not appear in the experimental group until they had achieved more than twice the average body weight at which it had been observed in the controls.

Although the differences observed following maternal restriction during gestation alone were smaller in magnitude, their implications are clearer and perhaps more fundamental. The results of these latter two experiments, taken together, strongly suggest that the subsequent development of the rat fetus can be affected by maternal nutritional deficiency during pregnancy. The fact that the pups were fostered shortly after birth to mothers which had been adequately fed throughout pregnancy permits the inference that maternal undernutrition had induced a direct effect upon the fetus. It is quite possible that dietary restriction during pregnancy causes a depletion of maternal reserves which is not immediately corrected by the restoration of adequate feeding at delivery with a consequent adverse effect on lactation. If pups

[6] See footnote 1.

are nursed by their own mothers it is not therefore possible to attribute subsequently observed abnormalities to damage sustained during fetal life.

The results appear to have other important implications. The very widely applied index of body weight would have revealed no impairment in the RN progeny, which were statistically indistinguishable by this criterion throughout the period of observation (even though they subsequently failed to match the growth of their controls). It is clear from these experiments that normal growth per se does not exclude the presence of damage from an earlier nutritional stress.

The observed delays in the neuromotor development of the experimental progeny also cast doubt on the absolute value of neurological indexes in the clinical determination of chronological age from conception. The recent recognition of the importance of distinguishing the infant born before term (true premature) from the undergrown infant born near term (dysmature) emphasizes the need for objective criteria of chronological age in the neonate. The development of the central nervous system has been thought to bear a close relationship to chronological age and has therefore been regarded as one of the best measures of time since conception. Most of the evidence for this supposition, however, has been obtained from infants whose intrauterine and early postnatal development had taken place under more or less satisfactory conditions. The fact that an infant born at 34 weeks of gestation achieves, at 6 weeks after birth, approximately the same level of neuromotor integration ordinarily shown at birth by a full-term infant does not in itself imply that the level of integration is independent of the nutritional experience.

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. Lee, C. J., and B. F. Chow 1965 Protein metabolism in the offspring of underfed mother rats. J. Nutr., *87:* 439.
3. 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.
4. Venkatachalam, P. S., and K. S. Ramanathan 1966 Severe protein deficiency during gestation in rats on birth weight and growth of offspring. Indian J. Med. Res., *54:* 402.
5. Venkatachalam, P. S., and K. S. Ramanathan 1964 Effect of protein deficiency during gestation and lactation on body weight and composition of offspring. J. Nutr., *84:* 38.
6. Caldwell, D. F., and J. A. Churchill 1967 Learning ability in the progeny of rats administered a protein-deficient diet during the second half of gestation. Neurology, *17:* 95.
7. Cowley, J. J., and R. D. Griesel 1966 The effect on growth and behaviour of rehabilitating first and second generation low protein rats. Anim. Behav., *14:* 506.
8. Frankova, S. 1965 Exploration activity of rats subjected to different types of stimuli. Cesk. Psychol., *1:* 58.
9. Lat, J., E. M. Widdowson and R. A. McCance 1960 Some effects of accelerating growth. III. Behavior and nervous activity. Proc. Roy. Soc. (London) Series B, *153:* 347.

Effect of Prior Diet on Lipid Mobilization in Rats During Starvation or Exposure to Cold

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ABSTRACT Using difference in the lipid content of the epididymal fat pads of the rat as a measure of depot fat changes, an attempt has been made to evaluate the role of previous feeding in the movement of fat out of adipose tissue under the stimuli of fasting or exposure to cold. There was a decreased rate of mobilization when the purified diet, consisting of vitamin-free casein as the protein source, was fed. Rats fed purified diet, however, mobilized fat at a rate equivalent to those fed Purina Laboratory Chow when the animals were exposed to 5°. The results indicate that rats fed a purified diet consisting of vitamin-free casein as the protein source mobilize significantly less adipose tissue fat during starvation than did control animals fed a diet of Purina Laboratory Chow; however, the feeding of the purified diet had no influence on the mobilization of lipid during exposure of the animal to 5°. Accordingly, alterations in adipose tissue metabolism which are insensitive to the effect of fasting appear to result from different mechanisms than those which determine the metabolic changes in this tissue subsequent to exposing the animal to a cold environment. The mechanism responsible for the activation of lipase during starvation remains unexplained.

Adipose tissue is exquisitely responsive to changes in the physiologic state of the organism. The importance of the process of lipid mobilization in providing a source of energy to the working cell is well established. Energy is stored in adipose tissue as esterified fatty acids in the form of triglycerides. It is made available to other tissues and organs in the intact organism by the release of free fatty acids (FFA) which circulate as fatty acid albumin complexes in plasma (1, 2). The mechanisms which control FFA release, however, are imperfectly understood. The mobilization of free fatty acids can be stimulated by several conditions including starvation (3, 4) and exposure to cold (5–7). The increased mobilization of FFA in cold-exposed rats would seem to be under the control of the sympathetic nervous system (8, 9). There is little or no evidence, however, that the sympathetic nervous system plays a role in initiating lipid mobilization during starvation.

Previous investigations have shown that diet composition has a pronounced effect on lipid metabolism (10–13). These investigators have placed considerable emphasis on lipogenesis, whereas relatively little attention has been paid to their effects on mobilization of lipids from fat depots. The present investigation was designed to explore this facet of the problem. Using differences in the lipid content of the epididymal fat body of the rat as a measure of depot fat changes, an attempt has been made to evaluate the role of previous feeding in the movement of fat out of adipose tissue. By comparing changes in depot fat due to starvation with those occurring in response to cold exposure, it was possible to distinguish differences. Evidence is presented which supports the hypothesis that the mobilization of FFA from adipose tissue due to starvation is triggered by mechanisms which are different from those which activate the increased mobilization of FFA in cold-exposed rats.

MATERIALS AND METHODS

Male, weanling albino rats of the Sprague-Dawley strain, weighing between 40 and 50 g each, were housed in individual cages having raised floors in a temperature-regulated environment. The animals were fed ad libitum and were allowed continuous access to water throughout the experimental period. The study consisted of five

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separate experiments.[2] In the first experiment 60 rats were used. They were divided into two groups and housed at 25 ± 2°. Group 1 received Purina Laboratory Chow;[3] the other was fed purified diet no. 5 described in table 1. At the end of about 6 weeks, half the animals in each group were killed. Food was removed from the remaining animals, and after 4 days starvation they were killed.

Experiment 2 was designed to test the effect of fat in the purified diet. The animals maintained at 25° were divided into four groups of 16 rats each. Each group was fed one of the following purified diets: no. 1, no. 2, no. 3 or no. 4. At the end of 7 weeks half the animals were killed. The remaining rats were starved for 4 days and then killed.

To insure that sufficient methionine was in the diet, 16 rats maintained at 25° were fed purified diet no. 1 supplemented with 0.3% L-methionine. At the end of 4 weeks half were killed; the remaining animals were starved for 4 days and then killed.

In experiment 4, 96 rats were used. They were divided into two equal groups and housed at 25 ± 2°. One group was fed stock Purina Laboratory Chow; the other was given purified diet no. 1. After about 4 weeks, 24 rats from each group were removed and housed at 5 ± 1°. At the end of 1 week in the cold, eight rats from each group were decapitated, as well as eight rats from the corresponding diet groups kept at 25°; the tissues were immediately removed for analysis. The remaining animals were kept in the cold for an additional 6 weeks. At that time eight rats from each group and their 25° controls were killed. Food was then removed from the remaining eight rats in each group at both environmental temperatures, and after 3 days of fasting they also were killed.

In experiment 5, 12 rats maintained at 25° were divided into two groups. One group was fed the Purina diet, the other was given purified diet no. 1. After 4 weeks food was removed and O_2 utilization measured during a 3-day starvation period.

Tissue lipid analysis. Immediately after decapitation the epididymal fat pads and liver were totally excised and weighed. The tissues were extracted with chloroform–methanol (2:1, v/v) using 20 ml solvent/gram tissue. The extraction was accomplished by vigorous homogenization with a mixer.[4] The mixture was filtered through a fiber glass filter pad under positive N_2 pressure. The filter cake was reextracted and the filtrates combined. The combined chloroform – methanol extract was evaporated to dryness under vacuum in a rotary evaporator at 10 to 15°. The solid residue was dissolved in a minimum

[2] Experiments reported here conformed with the standards of the "Guide for Laboratory Animal Facilities and Care."

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

[4] Omni-Mixer Homogenizer, Ivan Sorvall Inc., Norwalk, Conn.

TABLE 1

Composition of purified diets

	Diet no.				
	1	2	3	4	5
Casein (purified-vitamin free)	14.0	14.0	14.0	14.0	22.0
Glucose monohydrate	24.0	22.3	20.6	15.7	21.0
Sucrose	24.0	22.3	20.6	15.7	21.0
Dextrin	23.0	21.4	19.8	14.6	21.0
Lard	3.75	7.5	11.25	22.5	3.75
Corn oil	1.25	2.5	3.75	7.5	1.25
Cellulose	4.6	4.6	4.6	4.6	4.6
Mineral mix [1]	4.0	4.0	4.0	4.0	4.0
Vitamin mix [2]	1.0	1.0	1.0	1.0	1.0
Choline chloride	0.4	0.4	0.4	0.4	0.4

[1] The mineral mix at 4% of the diet provided: (in grams per kilogram of diet) $CaCO_3$, 11.716; KH_2PO_4, 13.724; NaCl, 10.024; $MgSO_4 \cdot 7H_2O$, 3.992; $CaHPO_4 \cdot 2H_2O$, 0.1718; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.2489; $CuSO_4$, 0.0623; $MnSO_4 \cdot H_2O$, 0.0484; $ZnCl_2$, 0.0080; KI, 0.0002; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0010; and Na_2SeO_4, 0.0006.

[2] The vitamin mix added at 1% of the diet provided: (in IU per kilogram diet) vitamin A, 5,000; vitamin D, 500; *dl*-α-tocopheryl acetate, 100; (in milligrams per kilogram diet) menadione, 5; thiamine·HCl, 10; riboflavin, 20; niacin, 50; ascorbic acid, 200; pyridoxine·HCl, 10; p-aminobenzoic acid, 100; biotin, 0.5; Ca pantothenate, 50; folic acid, 2; inositol, 200; and vitamin B_{12}, 0.05.

amount of chloroform – methanol (2:1, v/v), and nonlipid contaminants removed by means of column chromatography on Sephadex G-25 (14). The lipid eluate from the Sephadex column was used for the gravimetric determination of total lipid. Thin-layer chromatographic analysis revealed that at least 95% of the lipid in the epididymal fat pads was in the form of triglycerides. No further fractionation was carried out on these samples.

Liver lipid fractionation. A sample of the lipid eluate obtained from liver lipid extraction was evaporated to dryness *in vacuo*, as described above, and the residue dissolved in a minimum volume of chloroform. The chloroform solution was used to separate neutral lipids and free fatty acids from phospholipids by column chromatography. Silicic acid [5] was thoroughly washed with chloroform–methanol (2:1, v/v) followed by methanol. A column (1.4 cm by 10 cm) was prepared by packing the silicic acid as a slurry in methanol under a slight nitrogen pressure. The column was then washed with chloroform. The lipid sample was applied in 2 ml of chloroform. Neutral lipids and free fatty acids were eluted with 100 ml chloroform. Phospholipids were then eluted with 50 ml chloroform–methanol (2:1, v/v) followed by 50 ml methanol. Thin-layer chromatography of the fractions showed that the chloroform eluate was free of phospholipid and the phospholipid fraction was free of neutral lipids and free fatty acids. The separate fractions were evaporated to dryness under vacuum in a rotary evaporator and the quantity of residue measured gravimetrically.

Metabolic rate measurements. The rats were placed in a glass metabolic cage,[6] which was connected to a Noyons Diaferometer.[7] The animals remained in the cage without food for a period of 72 hours. Periodically during this time O_2 utilization was determined by measuring the difference in the O_2 concentration of air entering and leaving the cage at a constant air flow of 0.861/minute. Measurements were made only when the animal was at rest.

RESULTS

Results obtained from the analysis of the epididymal fat pads from rats fed either a stock Purina diet or diet 5 (table 1) are presented in table 2. It should be noted that the animals fed the Purina diet lost a great deal more weight during the starvation period than did the animals on a purified diet. The lipid content of the epididymal fat pads from the rats fed the Purina diet after starvation was significantly decreased, whereas the loss of lipid for the purified diet group was quite small. When the lipid content of the epididymal fat pads was calculated on the basis of body weight of the animal, it was evident that the group fed Purina Laboratory Chow lost a disproportionate amount of fat compared with the group fed the purified diet.

The animals were matched by weight as nearly as possible, to minimize any effect that differences in weight might have on fat mobilization during starvation. This offered an opportunity to observe the effects the diets might have on lipid deposition. It was apparent, when we compared the tissue weights or lipid content of the tissues from the group fed the Purina diet with those from the group fed the purified

[5] Mallinckrodt, 100 mesh, Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Delmar Scientific Labs, Maywood, Ill.
[7] Kipp and Zonen, Delft, Holland.

TABLE 2

Effect of starvation on epididymal fat pad lipids [1]

	Purina Laboratory Chow		Purified diet [2]	
	Fed	Starved	Fed	Starved
Initial body wt, g	254 ± 8 [3]	255 ± 6	243 ± 8	256 ± 9
Wt loss, g		78 ± 4		46 ± 6
Tissue wt, g	2.03 ± 0.33	0.76 ± 0.12	1.91 ± 0.23	1.36 ± 0.15
Total lipid, g	1.60 ± 0.21	0.49 ± 0.11	1.57 ± 0.21	1.08 ± 0.14
Lipid, mg/100 g body wt	629 ± 92	273 ± 43	648 ± 93	515 ± 69

[1] Fifteen rats per group; animals were starved for a period of 4 days.
[2] Diet no. 5.
[3] Mean ± SE.

diets, that fat deposition in the epididymal fat pad was the same. As can be seen in table 3, however, increasing the fat content of the diet resulted in an increased deposition of fat in the tissues. Nevertheless, the composition of the purified diet had no significant effect on the subsequent mobilization of fat from the epididymal fat pads during starvation. The results tabulated in table 3 were obtained from rats fed one of the purified diets presented in table 1. The protein content was 14% and the fat content varied from 5% to 30%. In none of these groups did mobilization of fat due to starvation approach that observed in animals fed the Purina Laboratory Chow; nor did supplementation of purified diet no. 1 with L-methionine have any effect.

Table 4 shows the results of analysis of liver lipid composition from rats fed either the Purina Laboratory Chow or a purified diet (exp. 1). In addition to body weight loss, there was a decrease in liver weight accompanying starvation. Though the loss of body weight in animals fed the purified diet was less than that in the group fed the Purina diet, the loss in liver weight was the same in both groups. The liver lipid composition remained constant in both groups whether the animals were fed or starved. Furthermore, there was no difference between groups with respect to liver lipid composition.

A comparison of the effects of prefeeding either the Purina diet or the purified diet on fat mobilization from the epididymal fat pads can be made from the results presented in figure 1 (exp. 4). Mobilization of fat was stimulated by the 3-day starvation, exposing the rats to an environment of 5° for a period of 6 weeks, or both. The results indicated that at both temperatures, rats fed a purified diet consisting of vitamin-free casein as the protein source mobilized significantly less adipose tissue fat during the 3-day starvation than animals fed the Purina diet. Main-

TABLE 3

Effect of diet fat composition on mobilization of fat from epididymal fat pads during starvation

Purified diet no.	Fat in diet		Initial body wt	Wt loss	Lipid
	%		g	g	mg/100 g body wt
1	5	Fed	278 ± 3 [2]		772 ± 41
		Starved [3]	270 ± 4	51 ± 2	629 ± 79
2	10	Fed	279 ± 6		811 ± 70
		Starved	274 ± 4	49 ± 7	778 ± 98
3	15	Fed	274 ± 5		910 ± 82
		Starved	272 ± 3	55 ± 6	759 ± 60
4	30	Fed	275 ± 7		921 ± 92
		Starved	271 ± 5	50 ± 4	832 ± 53

[1] Eight rats per group.
[2] Mean ± SE.
[3] Animals were starved for a period of 4 days.

TABLE 4

Effect of starvation on liver lipid content [1]

	Purina Laboratory Chow		Purified diet [2]	
	Fed	Starved	Fed	Starved
Body wt, g	254 ± 8 [3]	177 ± 7	243 ± 8	210 ± 11
Liver wt, g	11.8 ± 1.4	6.1 ± 0.5	12.3 ± 1.1	6.4 ± 0.8
Neutral lipid, mg/g tissue	15.8 ± 1.1	17.7 ± 1.5	17.6 ± 1.7	18.6 ± 1.4
Phospholipid, mg/g tissue	35.9 ± 1.9	36.6 ± 2.0	34.8 ± 1.6	36.1 ± 2.1

[1] Fifteen rats per group.
[2] Diet no. 5.
[3] Mean ± SE.

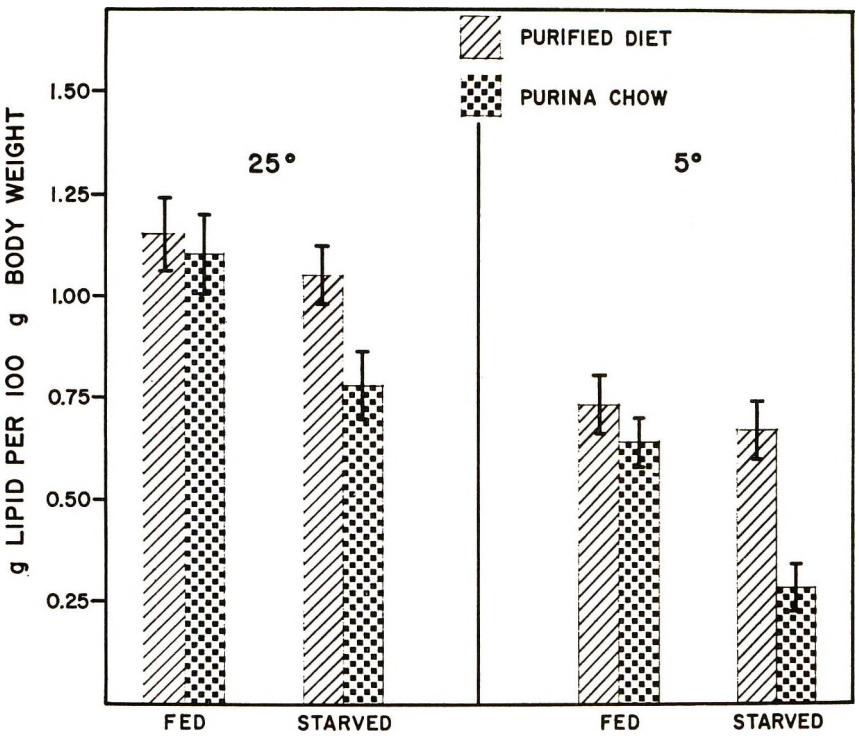

Fig. 1 Effect of diet on epididymal fat pad composition in fed or starved rats at either 25° or 5°. The bar graphs represent the mean ± SE of eight rats per group. Animals were maintained either at 25° or 5° for a period of 7 weeks. The group fed Purina Laboratory Chow at 25° weighed 398 ±27 g; those at 5° weighed 378 ± 31 g. The group fed the purified diet at 25° weighed 416 ± 36 g; those at 5° weighed 387 ± 21 g. Diet no. 1 was fed as the purified diet. Food was withdrawn from the starved groups 3 days before decapitation. All animals received water ad lib.

taining the rats in the cold resulted in fat pads which were considerably smaller than those from the 25° control groups, whether they were fed the Purina diet or the purified diet. It could not be determined if this was due to an increased mobilization of fat or to a decreased deposition in the cold. Table 5 presents the results obtained after an acute exposure to cold. It is seen that exposure to 5° for 1 week caused a marked mobilization of fat in both groups.

The decrease in O_2 utilization due to starvation is shown in table 6. Both groups of rats in the fed state had similar rates of O_2 utilization. Animals fed the purified diet, however, had a much lower resting metabolic rate at the end of the 72-hour fast than did the rats fed Purina Laboratory Chow. If we take into account the decreased weight of the animal during starvation and calculate the O_2 utilization per unit body mass, then it may be said that the metabolic rate of rats fed the Purina diet did not change during starvation. Even on the basis of body mass, however, the metabolic rate of rats fed the purified diet decreased approximately 35% during the 72-hour fast.

DISCUSSION

Changes in lipid metabolism in the intact animal are frequently measured by following the increase in concentration of FFA in plasma. This technique has certain obvious limitations. Although the increase in plasma FFA almost always indicated an increased liberation of FFA, the reverse is not necessarily true, since many stimuli increase both utilization and production simultaneously and may, therefore, cause

TABLE 5

Effect of cold on mobilization of fat from epididymal fat pads [1]

	Purina Laboratory Chow		Purified diet [2]	
	25°	5°	25°	5°
Body wt, g	236 ± 21 [3]	230 ± 27	190 ± 15	178 ± 12
Total lipid, g	1.96 ± 0.45	0.92 ± 0.23	1.61 ± 0.33	0.77 ± 0.15
Lipid, mg/100 g body wt	830 ± 157	400 ± 159	845 ± 165	436 ± 78

[1] Eight rats per group.
[2] Diet no. 1.
[3] Mean ± SE.

TABLE 6

Decrease resting O_2 utilization after starvation [1]

	Purina Laboratory Chow	Purified diet [2]
Initial O_2 utilization, mmoles/hour	14.1 ± 0.5 [3]	14.0 ± 0.6
Decrease after 72 hours, %	25.6 ± 8.1	46.6 ± 10.0
Initial body wt, g	210 ± 8	202 ± 6
Wt loss after 72 hours	48 ± 5	32 ± 4

[1] Six rats per group.
[2] Diet no. 1.
[3] Mean ± SE.

no change in the concentration, despite an increased turnover. Measurement of the disappearance of lipid from adipose tissue, on the other hand, is a convenient way to measure lipid mobilization in the intact animal, and does not suffer from this disadvantage. Because adipose tissue is quite diffuse throughout the body, it is difficult to recover it quantitatively from the rat. The epididymal fat pad, however, is a well-defined tissue, easily obtained in toto and therefore lends itself to the analysis of its lipid content. The assumption which is made is that the epididymal fat pad is representative of all adipose tissue.

Adipose tissue triglycerides constitute the major storage form of oxidizable substrate in mammals. The body's reserves of carbohydrate are very limited; consequently, during starvation depot fat is mobilized for dissemination to liver and peripheral tissues, as evidenced by a respiratory quotient approaching 0.7 (15). The data presented show that rats fed a purified diet consisting of vitamin-free casein as the protein source, mobilized significantly less adipose tissue fat during starvation than control animals fed a diet of Purina Laboratory Chow. However, the feeding of the purified diet had no influence on the mobilization of lipid due to exposure of the animal to an environment of 5°. It would appear, then, that a diet made up of purified components impairs the ability of the rat to mobilize adipose tissue lipid during periods of starvation, whereas the ability to mobilize lipid is not impaired during cold exposure. Accordingly, it may be concluded that adipose tissue metabolism which is affected by fasting results from different mechanisms than those which determine the metabolic changes in this tissue subsequent to exposing the animal to a cold environment.

It is not possible, from these results, to determine the reason for the inability of rats fed the purified diet to increase FFA mobilization during fasting. Results of liver lipid analysis (table 4) would indicate that it is not due to any abnormality of liver lipid metabolism. On the surface, it would seem that our observations are in conflict with results reported by DiGiorgio et al. (12). They observed that the diet composition had no significant effect on the in vitro release of FFA fom rat epididymal

fat pads, nor was total lipase activity affected. On closer examination, however, it is seen that these activities represent the fed state of the animal. In our present work, it was observed that the amount of lipid deposited in the epididymal fat pads was the same whether the animals were fed the Purina diet or the purified diet. If we consider that adipose tissue is in a dynamic state in which lipolysis is continuously going on, then it is reasonable to assume that in the fed state lipase activity is not affected by the diet. It is rather the ability of the animal to increase the release of FFA from adipose tissue which is affected by the feeding of a purified diet. Our observation that the purified diet did not inhibit the increased release of FFA in the cold-exposed rat is also in agreement with the results of DiGiorgio et al. (12), which show that in vitro norepinephrine-stimulated lipolysis is not significantly affected by the composition of the diet.

Obviously, it is difficult to consider biochemical mechanisms which regulate body fuel apart from the physiological states that these processes subserve in the organism. When a rat is exposed to cold, he is faced with a sudden need for additional energy to sustain a constant body temperature. There is an immediate activation of adipose tissue lipase (9, 16), and accompanying the increased mobilization of FFA there is a rise in metabolic rate (17, 18). This emergency mobilization of fuel energy is under the apparent precise control of norepinephrine released from the sympathetic nerves (8, 9, 19). When the rat is denied food, however, the increased mobilization of FFA which takes place is a gradual one. In addition, this increased output of FFA is accompanied by a decreased total expenditure of energy, as shown in table 5. Mobilization of fat from adipose tissue occurs primarily and probably exclusively in the form of FFA (2). In fact, Rizack (20) found that it is the "hormone-sensitive lipase" in adipose tissue which is activated in fasting rats. It is inconceivable that the activation of this enzyme is through sympathetic stimulation; if it were, we would expect to find an increase in O_2 utilization. Not only is there an absence of the calorigenic effect of norepinephrine, there is actually a decrease in total energy expenditure. This interpretation is consistent with earlier findings that the progressive increase in plasma FFA concentration that occurs during fasting is not prevented by the administration of ganglionic blocking agents (21–23). The mechanism responsible for the activation of lipase during starvation remains unexplained.

LITERATURE CITED

1. Gordon, R. S., Jr., and A. Cherkes 1956 Unesterified fatty acids in human blood plasma. J. Clin. Invest., *35:* 206.
2. Dole, V. P. 1956 A relation between nonesterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest., *35:* 150.
3. Goodman, H. M., and E. Knobil 1961 Some endocrine factors in regulation of fatty acid mobilization during fasting. Amer. J. Physiol., *201:* 1.
4. Gordon, R. S., Jr., and A. Cherkes 1958 Production of unesterified fatty acids from isolated rat adipose tissue incubated in vitro. Proc. Soc. Exp. Biol. Med., *97:* 150.
5. Wertheimer, E., M. Hamosh and E. Shafrir 1960 Factors affecting fat mobilization from adipose tissue. Amer. J. Clin. Nutr., *8:* 705.
6. Hannon, J. P., and A. M. Larson 1962 Fatty acid metabolism during norepinephrine-induced thermogenesis in the cold-acclimatized rat. Amer. J. Physiol., *203:* 1055.
7. Mitchell, C. E., and B. B. Longwell 1964 Effect of fasting and feeding on release of free fatty acid by adipose tissue of the cold-acclimatized rat. Proc. Soc. Exp. Biol. Med., *117:* 593.
8. Brodie, B. B., and R. P. Maickel 1963 Role of the sympathetic nervous system in drug induced fatty liver. Ann. N. Y. Acad. Sci., *104:* 1049.
9. Gilgen, A., R. P. Maickel, O. Nikodijevic and B. B. Brodie 1962 Essential role of catecholamines in the mobilization of free fatty acids and glucose after exposure to cold. Life Sci., *1:* 709.
10. Williams, M. A., K. T. Tamai and D. J. McIntosh 1967 Effects of fasting on liver lipids in rats fed a purified diet. Biochim. Biophys. Acta, *137:* 187.
11. Jansen, G. R., C. F. Hutchison and M. E. Zanettti 1966 Studies on lipogenesis in vitro. Effects of dietary fat or starvation on conversion of (^{14}C) glucose into fat and turnover of newly synthesized fat. Biochem. J., *99:* 323.
12. DiGiorgio, J., R. A. Bonanno and D. M. Hegsted 1962 Effects of diet upon the in vitro metabolism of rat epididymal adipose tissue. J. Nutr., *78:* 384.

13. Hausberger, F. X., and S. Milstein 1955 Dietary effects on lipogenesis in adipose tissue. J. Biol. Chem., *214:* 483.
14. Therriault, D. G., and R. H. Poe 1965 The effect of acute and chronic cold exposure on tissue lipids in the rat. Can. J. Biochem., *43:* 1427.
15. Von Pettenkofer, M., and C. Voit 1966 Studies on the metabolism of normal man. Z. Biol. (Berlin), *2:* 459.
16. Hanson, P. G., and R. E. Johnson 1965 Variation of plasma ketone and free fatty acids during acute cold exposure in man. J. Appl. Physiol., *20:* 56.
17. Depocas, F., J. S. Hart and O. Heroux 1957 Energy metabolism of the white rat after acclimation to warm and cold environments. J. Appl. Physiol., *10:* 393.
18. Sellers, E. A., J. W. Scott and N. Thomas 1954 Electrical activity of skeletal muscle of normal and acclimatized rats exposed to cold. Amer. J. Physiol., *177:* 372.
19. Brodie, B. B., J. I. Davies, S. Hynie, G. Krishna and B. Weiss 1966 Interrelationships of catecholamines with other endocrine systems. Pharmacol. Rev., *18:* 273.
20. Rizack, M. A. 1961 An epinephrine sensitive lipolytic activity in adipose tissue. J. Biol. Chem., *236:* 657.
21. Goodman, H. M., and E. Knobil 1959 Effect of adrenergic blocking agents on fatty acid mobilization during fasting. Proc. Soc. Exp. Biol. Med., *102:* 493.
22. Stern, D., and R. P. Maickel 1963 Studies on starvation induced hypermobilization of free fatty acids. Life Sci., *2:* 872.
23. Pinter, E. J., and C. J. Pottee 1968 Some data on the metabolic function of the adrenergic nervous system in the obese and during starvation. J. Clin. Endocrinol. Metab., *28:* 106.

Studies on Orotic Acid Fatty Liver in Rats: Factors influencing the induction of fatty liver [1]

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ABSTRACT Orotic acid methyl ester, a more water-soluble derivative of orotic acid, when force-fed along with diet, or when administered intraperitoneally to fed rats, induced a fatty liver, increased hepatic glycogen and increased hepatic total uridine nucleotides. Hepatic ATP levels decrease when orotic acid is added to a purified diet and fed ad libitum or by force-feeding. In force-feeding experiments when control and experimental animals are killed 14 to 16 hours after the last tube-feeding, hepatic ATP levels are equally low in both groups. Under these conditions the levels in the control animals drop to the sustained low levels in the experimental animals. Rats fed a diet containing lactose, calcium lactate and orotic acid do not develop fatty liver, yet have relatively low levels of hepatic ATP. On the other hand, there are moderately high levels of hepatic uridine nucleotides which indicate that there is no significant interference with orotic acid utilization. Based on experiments with fasted and fed animals given orotic acid or orotic acid methyl ester, diet appears to play an important role in the utilization of orotic acid and in the maintenance of elevated hepatic uridine nucleotide levels. Using the luciferin-luciferase method for assay of hepatic ATP levels, it was found that the livers of rats fed orotic acid had a stabilizing effect on the luminescence due to ATP. UDP had an inhibitory effect on the luminescence due to ATP.

Orotic acid when added to a purified diet and fed to rats results in the accumulation of hepatic lipid (1–4). This accumulation has been found to be preceded by an elevation of hepatic uridine nucleotides along with a decrease in hepatic ATP (3, 5, 6). The significance of the relationship between the increase in uridine nucleotides and the decrease in ATP in the pathogenesis of lipid accumulation in the liver is not well understood. Circumstantial evidence, however, such as a drop in hepatic ATP concomitant with the accumulation of hepatic lipid, and the prevention of the lipid accumulation by the administration of compounds like adenine (2, 3), and 5-amino-imidazole carboxamide (AICA) (7), all of which elevate hepatic ATP, have suggested that the decrease in ATP concentration is of importance in the induction of lipid accumulation. A direct relationship between ATP drop and lipid accumulation, however, is not well established. Reversal of orotic acid fatty liver by adenine could be explained by the fact that adenine competes with orotic acid for PRPP (5), thus interfering with the utilization of orotic acid. It is known that orotic acid fatty liver can be reversed by withdrawing orotic acid from the diet. Therefore, it was of interest to determine hepatic ATP levels under conditions where orotic acid fatty liver is inhibited by compounds other than those which increase hepatic ATP. In this regard we have taken into consideration two observations: 1) lactose and calcium lactate when fed along with orotic acid prevent lipid accumulation (8), and 2) orotic acid when given in water, without food, to fasted rats does not induce a fatty liver (9).

The present report is concerned with studies designed to elucidate the relative importance of ATP in the pathogenesis of orotic acid fatty liver, and the reasons for the inhibition of fatty liver in cases when lactose and calcium lactate are given along with orotic acid or when orotic acid is given with water to fasted rats.

EXPERIMENTAL

Female rats of the Sprague-Dawley strain [2] weighing 140 to 150 g were used

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[2] Sprague-Dawley, Madison, Wis.

in all the experiments. Animals were housed in individual wire-mesh cages and had free access to water. The control basal diet was the same as that described earlier (9) and the experimental diets had the supplemented compounds added at the expense of sucrose. In some experiments 25% lactose and 5% calcium lactate were added to the control basal diet. Orotic acid [3] and orotic acid methyl ester [4] were added to the diet at 1% levels unless indicated otherwise. In the experiments in which the animals were force-fed, they were tube-fed three times daily at 9:00 AM, 1:30 PM and 6:00 PM, similar to the procedure described previously (9). The diets used for force-feeding were blended with distilled water so that each milliliter of diet mixture contained 0.7 g of diet. Animals were force-fed an average daily intake of 0.7 g/10 g initial body weight. In all experiments the animals were force-fed the control basal diet for 1 or 2 days to accustom them to the purified diet, and the following morning they were weighed and divided into groups. Several groups of three to seven rats, each of the same age and weight, were used in each experiment. Rats were killed by decapitation. Liver lipid and glycogen were determined as described previously (9, 10). Hepatic total uridine nucleotides were assayed by the method as outlined by Windmueller (11) in the purine-free acid extracts and expressed as optical density (OD), at 262 mμ. For hepatic ATP determination, the animals were killed by decapitation and a piece of liver (about 1 g) was rapidly removed, weighed and homogenized in 10 ml of 0.5 N perchloric acid (PCA). The PCA extracts were neutralized to pH 6.9 to 7.0 with KOH solution and then used for ATP assay. ATP was measured by the luciferin-luciferase method (12) using desiccated firefly lanterns.[5] The incubation mixture contained 4 mg $MgSO_4 \cdot 7\ H_2O$,0.4 ml of filtrate of freshly prepared 1.5% desiccated firefly lanterns in 0.1 M arsenate buffer, pH 7.5, and test sample or standard ATP in a final volume of 4 ml. The reaction was started by the addition of enzyme. Luminescence was measured after 20 seconds (time taken to obtain maximum luminescence) using a fluorimeter.[6]

RESULTS

Problems in assay of hepatic ATP levels using luciferin-luciferase method. While assaying liver ATP levels by the luciferin-luciferase method using crude extracts of desiccated lanterns of firefly tails, it readily became apparent that the livers of rats fed orotic acid reacted in a different manner than did either the livers of animals fed the control basal diet or the ATP standards. Figure 1 shows the decay curves of luminescence of liver extracts from animals fed the control basal diet and of experimental animals fed the 1% orotic acid diet and the standard ATP preparations (1.48 to 2.96 mμmoles). The luminescence was more stable, particularly during the 20- to 30-second period, in the samples from experimental livers than in those from control livers or from a standard ATP preparation. In all three cases the maximum readings were obtained after 20 seconds and then decreased progressively over the following 100-second period of measurement.

To determine why the perchloric acid extracts of liver of rats fed orotic acid responded differently from extracts of control rats as indicated in figure 1, we de-

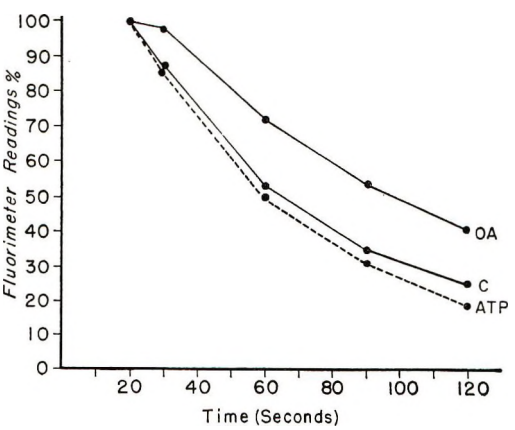

Fig. 1 Decay curves of luminescence due to ATP of liver extracts of rats force-fed 1% orotic acid (OA) diet or a control (C) diet for a period of 3 days, and of a standard ATP preparation. The results presented are the average of six determinations.

[3] Sigma Chemical Company, St. Louis, Mo.
[4] Cyclo Chemical Corporation, Los Angeles, Calif.
[5] See footnote 3.
[6] G. K. Turner Associates, Palo Alto, Calif.

cided to investigate whether uridine nucleotides, which became elevated in the livers of animals fed orotic acid, could be responsible. Our results indicated that UMP had little or no effect on the luminescence due to standard ATP even up to a UMP/ATP ratio of 1164. On the other hand, UDP was found to exhibit an inhibitory effect on the luminescence due to ATP (table 1). At high concentrations, UTP not only induced luminescence itself but also stabilized the luminescence when tested either alone or together with ATP.

Because of the different effects of uridine mono-, di- and triphosphates on the luciferin-luciferase system, the effects of other ribonucleotides were also tested on this system. AMP, only at high concentrations, inhibited the luminescence due to ATP. At a ratio of AMP/ATP of 535 (ATP concentration of 4.82 mμmoles) AMP completely inhibited the luminescence. Furthermore, addition of AMP increased the rate of decay of luminescence. GMP at a ratio of GMP/ATP of 510 and CMP at a ratio of CMP/ATP of 564 did not inhibit the ATP luminescence.

CDP and GDP, like UDP, inhibited the luminescence due to ATP (table 1). However, ADP, like ATP, gave luminescence with the homogenates of firefly lanterns. UDP which inhibits the luminescence due to ATP, did not inhibit the luminescence due to ADP even at a UDP/ADP ratio of 9.6. In these experiments 20.6 mμmoles of ADP were used. UDP has also been found to inhibit the luminescence due to UTP, CTP or GTP.

The luminescence obtained with a mixture of UTP and ADP was always greater than the sum of the activities obtained when UTP and ADP were assayed separately. These findings are in agreement with earlier results reported by Balfour and Samson (13) with other nucleoside triphosphates and have been attributed to the presence of nucleoside diphosphokinase.

Since our interests were primarily concerned with studies in rats fed an orotic acid diet, the possible direct effect of orotic acid on the assay system was also tested. Orotic acid (OA) and also orotic acid methyl ester (OAM), a more soluble compound, were found at ratios OA/ATP of 1652 and OAM/ATP of 1826 to inhibit luminescence (11 and 17%, respectively) at 20 seconds. These difficulties were overcome by using internal standards and by recording readings at 20 seconds when maximum luminescence was observed.

Effectiveness of orotic acid methyl ester on fed rats in inducing fatty liver when administered orally or intraperitoneally. Earlier studies (1–4) have demonstrated that 1% orotic acid in a purified diet induces fatty liver within days. These studies did not determine, however, whether orotic acid per se acted on the liver or whether it became altered within the gastrointestinal tract into an active metabolite which then acted on the liver to induce lipid accumulation. In our present study we used a more water-soluble form of orotic acid, orotic acid methyl ester, to investigate whether orotic acid could act

TABLE 1

Effect of UDP, CDP and GDP on luminescence due to ATP [1]

Nucleoside diphosphate (NDP) added to incubation mixture	NDP/ATP	Luminescence measured at 20 seconds		
		UDP	CDP	GDP
mμmoles		%	%	%
0	—	100	100	100
2.5	1.04	92	92	94
5.0	2.08	86	85	92
10.0	4.16	72	82	79
20.0	8.32	59	69	74
50.0	20.66	45	54	63
100.0	41.32	40	43	55
150.0	61.98	39	34	55

[1] The ATP used in these experiments ranged between 2.6 to 3.3 mμmoles.

directly to induce fatty liver. In a group of experiments this more soluble compound was given intraperitoneally shortly after each tube-feeding of the control basal diet, and a fatty liver was induced within 3 days (table 2). In addition, there was an increase in liver weight, glycogen and total uridine nucleotides with no change in liver ATP. In contrast, when this compound was tube-fed or given intraperitoneally to fasted rats, it did not induce fatty liver and did not cause a decrease, but rather an increase, in hepatic ATP levels (table 3). When orotic acid methyl ester was given with the control basal diet, however, either by stomach tube (table 3) or intraperitoneally (table 2), it caused an enlargement of the liver with increases in lipid, total uridine nucleotides and glycogen. This increase in hepatic glycogen was most probably related to the elevated hepatic uridine nucleotides. Thus, orotic acid methyl ester can act to induce fatty liver similar to that of orotic acid and can be effective when administered intraperitoneally or orally to fed rats.

Effect of lactose and calcium lactate on the induction of fatty liver by feeding orotic acid. Many studies have implicated ATP in the induction of orotic acid fatty

TABLE 2

Influence of lactose and calcium lactate in the diet along with orotic acid or orotic acid methyl ester on liver weight, lipid, glycogen, ATP and total uridine nucleotides of rats force-fed for 3 days

Group [1]	No. of rats	Liver wt	Liver lipid	Liver glycogen	Liver ATP	Liver total uridine nucleotides
		g/100 g body wt	*mg/liver per 100 g body wt*	*mg/liver per 100 g body wt*	*μmoles/liver per 100 g body wt*	*OD at 262 mμ/ liver per 100 g body wt*
C	9	4.5 ± 0.4 [2]	322 ± 36 [2]	163 ± 25 [2]	7.7 ± 0.7 [2]	51.9 ± 2.6 [2]
C + OA	10	5.9 ± 0.2 [3]	688 ± 66 [3]	378 ± 29 [3]	8.1 ± 0.4	137.7 ± 5.7 [3]
C + OAM	7	6.6 ± 0.3 [3]	537 ± 80 [4]	429 ± 22 [3]	8.3 ± 0.2	121.6 ± 11.3 [3]
CL	7	4.4 ± 0.2	317 ± 40	162 ± 33	5.1 ± 0.5	53.8 ± 5.6
CL + OA	7	5.0 ± 0.2	293 ± 29	275 ± 33	6.1 ± 0.4	97.0 ± 6.2 [3]
CL + OAM	6	5.5 ± 0.1	287 ± 29	355 ± 53 [4]	6.1 ± 0.7	109.0 ± 11.1 [3]

[1] C = control basal diet; C + OA = control basal diet containing 1% orotic acid; C + OAM = control basal diet and receiving 100 mg orotic acid methyl ester intraperitoneally in three equal doses 15 minutes after each tube-feeding; CL = control basal diet containing 25% lactose and 5% calcium lactate; CL + OA = CL diet containing 1% orotic acid; CL + OAM = CL diet and receiving 100 mg orotic acid methyl ester intraperitoneally in three equal doses 15 minutes after each tube-feeding.
[2] Mean ± SE of mean.
[3] $P < 0.01$.
[4] $0.05 > P > 0.01$.

TABLE 3

Effect of orotic acid methyl ester with or without diet for 3 days on hepatic weight, lipid, glycogen, ATP and total uridine nucleotides

Diet [1]	Orotic acid methyl ester [2]	No. of rats	Hepatic				
			Wt	Lipid	Glycogen	ATP	Total uridine nucleotides
			g/100 g body wt	*mg/liver per 100 g body wt*	*mg/liver per 100 g body wt*	*μmoles/liver/ 100 g body wt*	*OD at 262 mμ/ liver per 100 g body wt*
0	—	5		162 ± 9 [3]		3.64 ± 0.33 [3]	
0	+	5		154 ± 9		4.67 ± 0.23 [4]	
0	+ (ip)	5		183 ± 9		5.49 ± 0.29 [5]	
+ [6]	—	7	4.1 ± 0.1	290 ± 24	104 ± 20		42.0 ± 3.6
+	+	8	6.3 ± 0.2	763 ± 27 [5]	562 ± 11 [5]		140.3 ± 7.5 [5]

[1] Rats were tube-fed control basal diet with or without 1% orotic acid methyl ester three times daily.
[2] In experiment 1, 100 mg orotic acid methyl ester was administered either orally by stomach tube or intraperitoneally (ip) daily in three equally divided doses for 3 days. Animals were killed 4 hours after dose 3 on day 3. In experiment 2 the animals were tube-fed diets containing 1% orotic acid methyl ester for 3 days and killed on the morning of day 4.
[3] Mean ± SE of mean.
[4] $0.05 > P > 0.01$.
[5] $P < 0.01$.
[6] Experiment 2.

liver, because compounds that increase hepatic ATP inhibit the lipid accumulation due to orotic acid (2, 3, 7). A direct correlation between ATP and orotic acid fatty liver, however, is not yet established. Therefore, it was of interest to determine hepatic ATP levels under conditions where orotic acid fatty liver is inhibited by compounds other than those which increase hepatic ATP. In the present series of experiments the effects of lactose and calcium lactate on lipid accumulation, and changes in hepatic ATP and total uridine nucleotides induced by orotic acid were studied, since lactose and calcium lactate feeding was shown to inhibit fatty liver due to orotic acid (8). The results in table 2 clearly indicate that the addition of lactose and calcium lactate along with orotic acid in the diet inhibited the lipid accumulation in the liver, whether orotic acid was given either orally in the diet or as orotic acid methyl ester in solution intraperitoneally. The results in table 2 also indicate that the addition of lactose and calcium lactate to the diet containing orotic acid partially interferes wih the elevation of hepatic uridine nucleotides. This interference is somewhat less marked when orotic acid methyl ester was given intraperitoneally than when orotic acid was given orally. The animals fed the control diet containing lactose and calcium lactate had lower hepatic ATP levels than the animals fed the control basal diet (table 2). Furthermore, the results presented in table 2 indicate that when orotic acid was given either orally or as orotic acid methyl ester intraperitoneally along with the control basal diet or with the lactose and calcium lactate-supplemented diet it did not depress hepatic ATP concentration. These latter results are in agreement with the findings mentioned by Sidransky et al (9) using rats force-fed a purified diet containing orotic acid, but in disagreement with data reported by others using rats fed similar diets ad libitum (5, 6). To clarify the reason for this discrepancy, experiments were carried out in which animals were killed at different time intervals after the last tube-feeding of diet containing orotic acid methyl ester. Table 4 summarizes the results of these experiments which clearly indicate that at 4 and 8 hours after tube-feeding the orotic acid methyl ester containing diet there is a drop in hepatic ATP in comparison with controls. After 14 hours, however, the difference in ATP concentration between the animals fed the control diet and the experimental diet disappears, mainly due to a drop in the control animals. Animals fed ad libitum the same diet containing orotic acid

TABLE 4

Influence of time of feeding orotic acid diet on hepatic ATP levels

Group [1]	No. of rats	Time of killing after last feeding [2]	Hepatic ATP	
		hours	*μmoles/g liver*	*μmoles/liver per 100 g body wt*
C	8	4	2.17 ± 0.17 [3]	9.33 ± 0.94 [3]
OAM	8	4	1.59 ± 0.11 [4]	6.89 ± 0.08
C	4	8	2.17 ± 0.28	10.30 ± 1.43
OAM	4	8	1.26 ± 0.08 [4]	6.12 ± 0.13 [4]
C	9	14	1.61 ± 0.10	5.62 ± 0.45
OAM	9	14	1.53 ± 0.09	6.77 ± 0.43
C [5]	3	—	2.06 ± 0.27	10.11 ± 1.80
OA [5]	5	—	0.75 ± 0.05 [6]	5.61 ± 0.35 [4]

[1] C = control basal diet; OAM = 1% orotic acid methyl ester added to control basal diet; OA = 1% orotic acid added to control basal diet.

[2] Rats were killed at specified times after the third tube-feeding of diet. Rats received 10 g diet (control or experimental containing 100 mg OAM) in three divided feedings for 1 day.

[3] Mean ± SE of mean.

[4] $0.05 > P > 0.01$.

[5] Rats were fed diets ad libitum for 12 days and killed on the morning of day 13. Rats had access to diet until killing.

[6] $P < 0.01$.

for 12 days reveal a significant decrease in hepatic ATP (table 4).

Influence of diet on the induction of orotic acid fatty liver. In an earlier report (9) and confirmed in this study (table 3), it was found that orotic acid or orotic acid methyl ester did not induce a fatty liver when administered without diet. The following two factors in the fasting state were considered as of possible importance: 1) impairment of orotic acid utilization and consequently no decrease in hepatic ATP; and 2) impairment in the accumulation of hepatic lipid independent of the alterations in the nucleotide metabolism. The results in table 5 indicate that in the fasted state hepatic uridine nucleotides become maximally elevated after 8 hours but this high level is not maintained; also, there is no decrease, but rather an increase, in hepatic ATP (table 3). The results in table 5 further indicate that the livers of rats given orotic acid along with diet reach a somewhat higher level of total uridine nucleotides after 8 hours, and that this high level was maintained for at least 24 hours. The importance of the administration of diet together with orotic acid is emphasized in experiment C of table 5. Here the rats were tube-fed orotic acid in water and then were offered diet ad libitum 6 hours later and until they were killed after 24 hours. Hepatic total uridine nucleotides levels were not elevated in these experimental animals.

DISCUSSION

In the present series of experiments, attempts were made to correlate hepatic ATP levels with orotic acid fatty liver formation by inhibiting the fatty liver, with compounds or under conditions other than those that increase in vivo hepatic ATP levels. Our present results indicate that rats tube-fed a purified diet containing lactose and calcium lactate with orotic acid (table 2) do not develop fatty livers and confirms the earlier findings by Kobata et al. (8).

TABLE 5

Effect of orotic acid methyl ester with or without diet on hepatic total uridine nucleotides levels

Exp.	Diet [1]	Orotic acid methyl ester (OAM), 100 mg [2]	Time killed after administration of OAM	No. of rats	Liver total uridine nucleotides
			hours		*OD at 262 mμ/g liver*
A	0	−	0	3	12.3 ± 1.4 [3]
	0	+	1	3	15.9 ± 0.7
	0	+	2	3	15.7 ± 0.6
	0	+	4	3	17.2 ± 1.6
	0	+	8	3	18.6 ± 0.5
	0	+	12	3	16.0 ± 0.8
	0	+	24	3	14.3 ± 1.0
B	0	−	8	3	12.1 ± 0.9
	+	−	8	3	8.4 ± 0.9
	0	+	8	6	18.5 ± 0.5
	+	+	8	6	22.6 ± 1.0
	0	−	24	6	10.2 ± 1.1
	+	−	24	5	10.6 ± 1.6
	0	+	24	6	11.9 ± 1.1
	+	+	24	3	26.5 ± 1.6
C	0	−	24	3	11.5 ± 0.1
	+	−	24	3	13.6 ± 1.3
	0	+	24	3	11.6 ± 0.1
	+	+	24	3	14.5 ± 1.0

[1] In experiment B rats were tube-fed 4.7 g control basal diet containing orotic acid at zero time; in experiment C rats were offered control basal diet ad libitum 6 hours after receiving orotic acid.
[2] Orotic acid methyl ester, 100 mg in 5 to 7 ml distilled water or in control basal diet, was tube fed to rats.
[3] Mean ± SE of mean.

This is of interest because feeding lactose has been found to result in a decreased capacity to utilize glucose (14) which is important for ATP generation via the citric acid cycle. Under these conditions, interference with orotic acid absorption does not seem to be a major mechanism, since lactose and calcium lactate feeding inhibited fatty liver formation even when orotic acid methyl ester was given intraperitoneally. One possibility by which lactose and calcium lactate could act is by causing a decrease in glucose utilization, and thus leading to a decrease of acetyl-CoA which is a key substrate for fatty acid synthesis. If the increased lipid in rats fed orotic acid is largely synthesized from the dietary intake, as indicated by the observations of Windmueller (15), such inhibition of the formation of acetyl-CoA from glucose may tend to prevent the induction of fatty liver. However, since there is reported to be a block in lipid transport from the liver in orotic acid-fed rats (15), it is possible that the administration of lactose and calcium lactate acts in some unknown manner to remove this block in transport.

The present study clarifies the discrepancy between the earlier reports (5, 6) indicating a decrease in hepatic ATP concentrations in rats fed ad libitum an orotic acid diet and the report (9) of no decrease in hepatic ATP concentrations in rats force-fed a similar diet. When animals were force-fed an orotic acid diet to induce fatty liver, there was a 14- to 16-hour interval between the last feeding and the time of killing. During this interval there was a drop in hepatic ATP levels in the controls to the low levels already present in the experimental animals (table 4), so that, at the time of killing there was essentially no difference in hepatic ATP levels between the two groups. The failure to observe a decrease in hepatic ATP levels in the experimental tube-fed rats at 14 hours after the last feeding suggests that either further degradation of ATP is inhibited or its synthesis is accelerated to maintain a relatively constant, yet low, level of ATP. This latter possibility is consistent with the recent results of Windmueller and Spaeth (16) and Rajalakshmi and Handschumacher (17) who reported increased hepatic purine nucleotide synthesis in rats fed orotic acid. Also, since degradation of hepatic purine nucleotides in animals fed orotic acid is greater than in controls (6), the failure to observe a decrease in hepatic ATP 14 hours after the last feeding of orotic acid diet suggests increased ATP synthesis.

In an earlier study (9) it was shown that orotic acid must be given together with diet to induce fatty liver. Some of the possible explanations for this could be that orotic acid was not properly metabolized in the fasted state or that dietary components, such as carbohydrates, were essential for the increase in lipid accumulation. In consideration of the former possibility, experiments were conducted with fasted rats and the results indicate that orotic acid methyl ester when tube-fed or given intraperitoneally did not cause a drop in hepatic ATP in such animals (table 3). Even though fasted animals showed a moderate increase in hepatic uridine nucleotides after tube-feeding orotic acid methyl ester, the level dropped within 24 hours. This was in contrast to the fed animals where the level was maintained up to at least 24 hours (table 5). That diet is intimately related to the time of administration of orotic acid is further emphasized by the findings that fasted rats given orotic acid methyl ester by stomach tube and then given diet ad libitum 6 hours later did not show an elevated level of hepatic uridine nucleotides (table 5). Thus, it appears that diet may play at least two important roles in the induction of fatty liver due to orotic acid: 1) it may influence the formation and maintenance of elevated hepatic uridine nucelotides, especially that of UMP, which has been shown to stimulate fatty acid biosynthesis in in vitro conditions,[7] and 2) it may supply essential dietary components such as carbohydrates which are necessary for the lipid accumulation.

Our present observations that fatty liver can be induced by orotic acid methyl ester when administered intraperitoneally to fed animals is of importance. It indicates that the compound can act directly upon the liver. Earlier studies (1–9) using orotic

[7] Sarma, D. S. R., and H. Sidransky 1967 Studies on orotic acid fatty liver: Influence of uridine-monophosphate (UMP) on fatty acid synthesis. Federation Proc., 26: 624 (abstract).

acid in the diet had not ruled out that orotic acid may become altered in the gastrointestinal tract into an active metabolite which then induces fatty liver. Windmueller et al (18) reported that orotic acid in the diet could induce fatty liver even in germfree rats, suggesting that the intestinal bacterial flora did not play a role in this process. Further support for the position presented of the direct action of orotic acid on the liver to induce lipid accumulation comes from a report by Von Euler and Windmueller (19) that the continuous intravenous infusion of the ammonium salt of orotic acid to fed rats induced fatty liver.

In our present investigation we encountered problems using the luciferin-luciferase method for assay of hepatic ATP levels which were overcome by using internal standards. Because nucleotide levels in tissue, particularly liver, become altered in a variety of experimental conditions, one of which is illustrated in our present study, it is important to be aware that such alterations in pool sizes can play an important part in the quantitative assay of tissue ATP level using the luciferin-luciferase method. Therefore, caution must be exercised when one uses homogenates of firefly lanterns for ATP determinations on biological material.

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LITERATURE CITED

1. Standerfer, S. B., and P. Handler 1955 Fatty liver induced by orotic acid feeding. Proc. Soc. Exp. Biol. Med., *90:* 270.
2. Handschumacher, R. E., W. A. Creasey, J. J. Jaffer, C. A. Pasternak and L. Hankin 1960 Biochemical and nutritional studies on the induction of fatty liver by dietary orotic acid. Proc. Nat. Acad. Sci. US, *46:* 178.
3. Rajalakshmi, S., D. S. R. Sarma and P. S. Sarma 1961 Studies on orotic fatty liver. Biochem. J., *80:* 375.
4. Sidransky, H. 1963 Sex difference in induction of fatty liver in the rat by dietary orotic acid. Endocrinology, 72: 709.
5. Von Euler, L. H., R. J. Rubin and R. E. Handschumacher 1963 Fatty liver induced by orotic acid. II. Changes in nucleotide metabolism. J. Biol. Chem., 238: 2464.
6. Marchetti, M., P. Puddu and C. M. Caldarera 1964 Metabolic aspects of orotic acid fatty liver: Nucleotide control mechanisms of lipid metabolism. Biochem. J., 92: 46.
7. Hayano, K., T. Tsubone, I. Azuma and Y. Yamamura 1962 The prevention of the orotic acid fatty liver by the administration of 5-amino-imidazole carboxamide. J. Biochem., 52: 379.
8. Kobata, A., K. Furuno and Z. Suzuoki 1964 The chemical components of milk which prevent the fatty liver induced by orotic acid. J. Biochem., 55: 92.
9. Sidransky, H., E. Verney and B. Lombardi 1963 Factors influencing the induction of fatty liver by orotic acid. J. Nutr., *81:* 348.
10. Sidransky, H., E. Verney and D. S. Wagle 1965 Effect of dietary orotic acid on liver glycogen. Proc. Soc. Exp. Biol. Med., *120:* 408.
11. Windmueller, H. G. 1965 Hepatic nucleotide levels and NAD-synthesis as influenced by dietary orotic acid and adenine. J. Nutr., *85:* 221.
12. Stehler, B. L., and W. D. McElroy 1957 In: Methods in Enzymology, vol. 3, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 871.
13. Balfour, W. M., and F. E. Samson, Jr. 1959 Transphosphorylasis in the firefly lantern. Arch. Biochem. Biophys., *84:* 140.
14. Bernhart, F. W., E. D'Amato and R. M. Tomarelli 1961 Glucose uptake by epididymal fat pads from rats fed glucose, lactose or a glucose-galactose mixture. Proc. Soc. Exp. Biol. Med., *107:* 551.
15. Windmueller, H. G. 1964 An orotic acid induced and adenine reversed inhibition of hepatic lipoprotein secretion in the rat. J. Biol. Chem., *239:* 530.
16. Windmueller, H. G., and A. E. Spaeth 1965 Stimulation of hepatic purine biosynthesis by orotic acid. J. Biol. Chem., *240:* 4398.
17. Rajalakshmi, S., and R. E. Handschumacher 1968 Control of purine biosynthesis de novo by orotic acid in vivo and in vitro. Biochim. Biophys. Acta, *155:* 317.
18. Windmueller, H. G., E. G. McDaniel and A. Spaeth 1965 Orotic acid-induced fatty liver: Metabolic studies in conventional and germ-free rats. Arch. Biochem. Biophys., *109:* 13.
19. Von Euler, L. H., and H. G. Windmueller 1967 Fatty liver in the rat liver after intravenous infusion of orotic acid. Proc. Soc. Exp. Biol. Med., *125:* 1251.

Circulating Antibody Formation in Scorbutic Guinea Pigs [1]

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ABSTRACT Severe scurvy was induced in young guinea pigs by feeding a highly purified ascorbic acid-deficient diet. After immunization with diphtheria toxoid, no deleterious effects on primary or secondary circulating antibody formation were observed in these scorbutic guinea pigs. A reduction in the early, Arthus-type, dermal hypersensitivity observed in the immunized scorbutic guinea pigs did not appear to be immunological in nature.

The role of ascorbic acid in the mechanisms of antibody synthesis has long been a matter of dispute. Conflicting results of various workers have made it difficult to arrive at any clear-cut correlation (1, 2). In one of the more definitive studies in this area, Long (3) observed a deleterious effect on the secondary response of circulatings antibodies in guinea pigs fed an ascorbic acid-deficient natural diet for a prolonged period of time. Using a highly purified diet, we have previously observed that Vitamin B_6-deficient guinea pigs had a depressed circulating antibody formation to the antigenic stimulus of diphtheria toxoid (4). As a continuation of an extensive program in our laboratories concerned with the role of vitamins in the development of immune processes, we have undertaken the present study on the effects of ascorbic acid deficiency produced by a highly purified diet.

MATERIALS AND METHODS

Animals. Male, albino guinea pigs of the Hartley strain, weighing 300 to 450 g, were kept in individual cages with wide-meshed screen bottoms and weighed weekly.

Diets. A highly purified diet (no. 13) described by Reid and Briggs (5) was used with the modification that ascorbic acid was omitted and Alphacel [2] was substituted for cellophane spangles. This diet (table 1) is designated in this paper as the basal diet. Supplementary ascorbic acid was administered daily by pipette as 0.5 ml/animal of a freshly prepared aqueous solution containing 5 mg of L-ascorbic acid. Animals were fed the basal diet ad libitum unless indicated otherwise. All guinea pigs were fed the purified basal diet supplemented with ascorbic acid for 1 week following their receipt from the suppliers. Experimental procedures were started immediately after this period of adaptation.

Immunization and serum antibody titration. Guinea pigs were immunized by intraperitoneal injection of 0.15 ml of an alum-precipitated diphtheria toxoid preparation containing 50 Lf units/ml.[3] Animals were bled by cardiac puncture under ether anesthesia 3 weeks later and the primary response of circulating (serum) antibodies was determined by Stavitsky's modification (6) of the Boyden (7) tanned erythrocyte technique. An identical dosage of the diphtheria toxoid was then administered as described and animals bled by cardiac puncture 4 to 5 days later for the determination of the secondary (booster) circulating antibody response by the same technique. Skin tests to determine the degree of hypersensitivity in immunized animals were performed at this time.

Skin tests. Immunized guinea pigs were injected intradermally with 0.2 ml of a formol diphtheria toxoid preparation containing 40 Lf units [4] on one side of the back

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[2] Alphacel was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Kindly supplied by Eli Lilly and Company, Indianapolis, Ind.

[4] See footnote 3.

TABLE 1

Composition of basal diet

Ingredients	
	g/kg
Casein (vitamin-free) [1]	300
Corn oil	73
Sucrose	103
Alphacel [1]	150
Starch (corn)	200
Glucose	78
Potassium acetate	25
Magnesium oxide	5
Salts, Briggs [1]	60
Choline chloride	2
Inositol	2
	mg/kg
Thiamine·HCl	16.00
Riboflavin	16.00
Pyridoxine·HCl	16.00
Calcium pantothenate	40.00
Niacin	200.00
Biotin	0.60
Folic acid	10.00
Vitamin B_{12}	0.04
Vitamin A acetate	6.00
Vitamin D_3	0.04
2-Methyl-1,4-naphthoquinone	2.00
Alpha-tocopheryl acetate	20.00

[1] Purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

and with 0.1 ml of an aqueous solution containing 275 μg histamine phosphate on the other. In addition, occasional animals were injected intradermally with 0.2 ml of physiological saline. At varying times thereafter, thickness and diameter of the inflamed area was determined as described earlier (4).

RESULTS

Primary immune response to diphtheria toxoid in scorbutic guinea pigs. In this experiment, animals were grouped as indicated in table 2. Ascorbic acid deficiency was induced by feeding the basal diet during the 4-week experimental period, whereas both control groups, receiving the same basal diet, were given daily, oral supplements of ascorbic acid during this period. It was necessary to include a control inanition (pair-fed) group since food intake of the ascorbic acid-deficient animals was markedly diminished. Throughout the 4-week experimental period, each animal in the inanition control group received an amount of basal diet equal to that consumed by its ascorbic acid-deficient partner during the previous day.

After 1 week each guinea pig was immunized with diphtheria toxoid; 3 weeks later all were bled for determination of the primary response. The high mortality rate observed in guinea pigs fed an ascorbic acid-deficient diet for 3 to 4 weeks precluded the primary administration of antigen in later stages of the deficiency.

It is apparent (table 2) that guinea pigs fed the basal ascorbic acid-deficient diet exhibited a marked loss in weight. Disseminated hemorrhagic areas characteristic of scurvy were noted on autopsy. All these symptoms of scurvy could be cured solely by therapy with ascorbic acid for 2 to 3 weeks. Clearly, a specific and severe ascorbic acid deficiency had been produced in these guinea pigs.

Despite the severity of their deficiency state, the ascorbic acid-deficient animals were capable of a normal primary antibody response to the antigen, diphtheria toxoid, as determined by the tanned erythrocyte technique (table 2). The lack of a depressant effect of inanition per se upon antibody response (table 2) is in agreement

TABLE 2

Serum antibody titers of guinea pigs after primary immunization with diphtheria toxoid

Group	Initial body wt [1]	Final body wt [1]	Titers [2]
	g	*g*	
Control, ad lib fed	409	466	1:640
	374	500	1:320
	380	493	1:640
	354	450	1:320
	426	508	1:640
Control, pair-fed [2]	420	380	1:160
	476	450	1:320
	409	460	1:320
	472	430	1:320
Ascorbic acid deficient	409	250	1:640
	472	280	1:320
	440	270	1:320
	465	350	1:320
	449	350	1:80
	489	330	1:320

[1] Initial weights were determined at the beginning of the experimental period and final weights 4 weeks later at the time of bleeding.

[2] Agglutinating antibody titers of serum and values for individual animals presented in this table were determined by the tanned erythrocyte technique. In this method, determinations are made of final serum dilutions capable of agglutinating tanned erythrocytes upon which the antigen (diphtheria toxoid) has been absorbed. Thus, the magnitude of the titer (serum dilution) is a direct measure of the content of serum agglutinating antibodies.

with many previous observations in our laboratory.

Secondary immune response to diphtheria toxoid in scorbutic guinea pigs. Diets utilized in the control group fed ad libitum and the ascorbic acid-deficient group, as well as methodologies for the immunization and determination of the secondary circulating antibody response and early, Arthus-type skin activity response, have been described in Materials and Methods. The primary injection of diphtheria toxoid was administered to control animals on day 1 of the experimental period; the animals were maintained on this dietary regimen during the entire experimental period of 25 to 26 days. To ensure an adequate number of survivors for determination of the anamnestic (booster) response, the following experimental plan was adopted for the ascorbic acid-deficient group. The primary injection of diphtheria toxoid was administered on day 1 of the experimental period and the guinea pigs continued to receive the daily, oral supplement of ascorbic acid for 1 week. Thereafter, supplementation of ascorbic acid was omitted for the remainder of the experimental period (18 to 19 days). Animals on this regimen lost weight and exhibited severe symptoms of scurvy at the time of bleeding (fig. 1). Values for the secondary circulating antibody response are shown in figure 1. Primary responses were not determined in this experiment and only secondary responses are presented. As measured by the tanned erythrocyte technique, no effect of this severe ascorbic acid deficiency state upon the ability of guinea pigs to establish an anamnestic response to diphtheria toxoid was evident (fig. 1). Skin reactions measuring degree of hypersensitivity of the early Arthus-type induced by immunization with diphtheria toxoid are presented in figure 2. A reduction in degree of this reaction appeared in the scorbutic guinea pigs (fig. 2). However, a similar reduction in the reaction of scorbutic guinea pigs to the nonspecific irritant, histamine, was also observed.

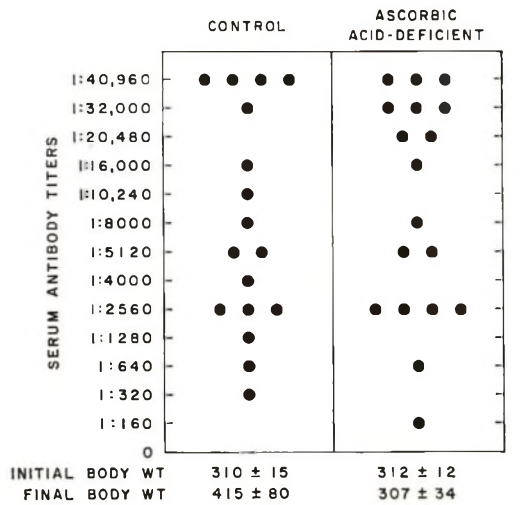

Fig. 1 Scatter diagram of secondary circulating antibodies of scorbutic guinea pigs. A booster injection of diphtheria toxoid was administered 3 weeks after the primary injection and the animals bled for determination of the anamnestic response 4 to 5 days later. Each point represents serum titer of an individual animal. Initial weights were determined at the beginning of the experimental period and final weights at the time of bleeding. Body weight is expressed in grams as average ± SE.

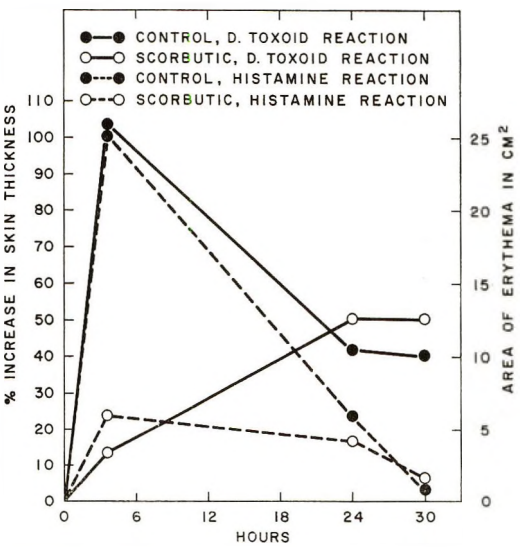

Fig. 2 Hypersensitivity in guinea pigs immunized with diphtheria toxoid as measured by skin reactivity to intradermally injected diphtheria toxoid. Reactions to diphtheria toxoid are given as percentage increase of skin thickness; the histamine reaction is given as areas of erythema since, in this case, changes in skin thickness were too small for accurate measurement.

DISCUSSION

In the present study we have failed to observe any deleterious effects on antibody production in guinea pigs with a severe de-

ficiency of ascorbic acid. In this respect, our findings differ from those reported by Long (3). However, a direct comparison of our results with those reported by Long is difficult because many differences in methodology were used in these two studies. We used a highly purified diet which induced a severe deficiency state; Long used a diet composed of natural foodstuffs containing variable small amounts of ascorbic acid which, by the criteria of growth inhibition and macroscopic morphological changes, did not produce a severe ascorbic acid deficiency. Subsequent observations have indicated that the diet used by Long was also deficient in methionine and produced an abnormally low sulfhydryl content in tissues.[5] Both the dosage and route of administration of diphtheria toxoid also differed in the two studies. Perhaps the greatest variation in technique lay in the method of estimating the serum antibody level. Long used the intradermal method of Römer and Sames (8) which is based on the specific neutralization of diphtheria toxin by serum diphtheria antioxin, whereas we used a passive hemagglutination technique which most probably assays a complex of antibodies resulting from the stimuli of a variety of antigens present in the diphtheria toxoid preparation. It is of some interest to note that Stavitsky (9) reported a "poor and inconsistent" correlation between these two methods in guinea pig sera assayed at the levels of antitoxin present in these two studies.

The reduction in the early Arthus-type skin reactions observed in the immunized, scorbutic animals does not appear to be related to a decreased immune response since a similar reduction in reactivity to the nonspecific irritant, histamine, was also noted in scorbutic guinea pigs. Zweiman et al. (10) have also reported a decreased response to the nonspecific irritant, benzyl alcohol, in scorbutic guinea pigs.

In conjunction with the lack of effect of ascorbic acid deficiency upon circulating antibody production, we did not observe any difference between the total amino acid incorporating capacities of microsomes from the spleens of normal (7400 cpm) and scorbutic (7000 cpm) immunized guinea pigs.[6]

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Axelrod, A. E., and J. Pruzansky 1955 Role of the vitamins in antibody production. Vitamins Hormones, *13:* 1.
2. Axelrod, A. E. 1968 Nutrition in relation to acquired immunity. In: Modern Nutrition in Health and Disease, Ed. 4, eds., M. G. Wohl and R. S. Goodhart. Lea and Febiger, Philadelphia, p. 612.
3. Long, D. A. 1950 Ascorbic acid and production of antibody in the guinea pig. Brit. J. Exp. Pathol., *31:* 183.
4. Axelrod, A. E., S. Hopper and D. A. Long 1961 Effects of pyridoxine deficiency upon circulating antibody formation and skin hypersensitivity reactions to diphtheria toxoid in guinea pigs. J. Nutr., *74:* 58.
5. Reid, M. E., and G. M. Briggs 1953 Development of a semi-synthetic diet for young guinea pigs. J. Nutr., *51:* 341.
6. Stavitsky, A. B. 1954 Micromethod for the study of proteins and antibodies. J. Immunol., *72:* 360.
7. Boyden, S. V. 1951 Absorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera. J. Exp. Med., *93:* 107.
8. Römer, P. H., and T. Sames 1909 Determination of very small quantities of diphtheria antitoxin. Z. Immunitatsforsch. Exp. Therap., *3:* 344.
9. Stavitsky, A. B. 1954 Micromethod for the study of proteins and antibodies. J. Immunol., *72:* 368.
10. Zweiman, B., W. F. Schoenwetter and E. A. Hildreth 1966 The effect of the scorbutic state on the tuberculin hypersensitivity in the guinea pig., J. Immunol., 96: 296.

[5] Long, D. A., personal communication.
[6] Kumar, M., and A. E. Axelrod, unpublished observations.

Effects of Severe Alkali Treatment of Proteins on Amino Acid Composition and Nutritive Value [1]

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ABSTRACT Amino acid analyses, protein quality assays, in vitro digestion and absorption tests and feeding studies with rats were conducted to study the effects of alkali treatment on food proteins under varying conditions of pH, temperature and time. Exposure of several high protein products to aqueous alkali at pH 12.2 resulted in the formation of the amino acid derivative lysinoalanine (LAL) which is poorly absorbed. The amount of LAL formed in isolated soy protein (ISP) upon exposure at pH 12.2 increased with rising temperature and a longer exposure period. The presence of LAL in proteins was attended with decreased contents of cystine and lysine, and decreased net protein utilization (NPU) values. More severe treatment of ISP with alkali of pH 12.2 at 60 or 80° also caused a decrease in serine content and in digestibility of the protein. The LAL content of ISP treated under various conditions showed a highly significant negative correlation with NPU values ($r = -0.96$). The presence of LAL in proteins was a sensitive criterion of alkali damage. The NPU assays of ISP supplemented with amino acids showed methionine to be the first limiting amino acid and threonine the second. The decreased NPU of alkali-treated ISP could not be completely alleviated by amino acid supplementation, probably as a result of decreased utilization of threonine. Most of the essential amino acids from alkali-treated ISP were released at a relatively slow rate by pepsin-pancreatin digestion. Threonine and methionine were the only essential amino acids in an enzymatic digest of ISP which showed decreased absorption by everted intestinal sacs. Upon feeding rats diets with relatively high levels of proteins treated at pH 12.2 and 40° for 4 hours no clinical or histological abnormalities were observed other than an increased degree of nephrocalcinosis in females which could be prevented by additional dietary calcium.

Exposure of proteins to alkali is increasingly applied in technological treatment of foods and feeds, e.g., for dissolving proteins in the preparation of concentrates and isolates [2] (1); for obtaining proteins with specific properties such as foaming, emulsifying or stabilizing (2); for destruction of aflatoxin in groundnuts (3); and for obtaining protein solutions suitable for spinning fibers (4). Several authors have observed that alkali treatment of wool, enzymes and serum albumin may induce chemical changes in these proteins which lead to the formation of new amino acids: lysinoalanine (5, 6), lanthionine (7) and ornithinoalanine (8). These modifications involve the amino acids cystine, lysine, arginine and possibly serine.

During routine analyses of amino acids in proteins Slump [3] observed an unknown peak in the chromatograms of alkali-treated foods and feeds which upon identification turned out to be lysinoalanine.[4] Obviously alkali treatment of food proteins may result in chemical changes similar to those mentioned above and very likely affecting the same amino acids, e.g., cystine and lysine. Since these are the limiting amino acids in the majority of food proteins, an impaired nutritive value may result from exposure to alkali. A study was undertaken, therefore, to evaluate the effects on food proteins, of alkali treatments varying in pH, temperature and duration, by amino acid analysis, biological assays of protein quality and in vitro tests of digestion and absorption. In addition, attention was paid to possible harmful properties of alkali-treated proteins by feeding drastically treated proteins at relatively high levels

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[1] Presented in part at the 9th International Symposium of the Commission Internationale des Industries Agricoles et Alimentaires (C.I.I.A.) on "New protein sources in the human diet," Amsterdam, November, 1968.

[2] Galliver, G. B., and A. W. Holmes 1959 Protein containing food product from fish. Assignors to Unilever N.V., Rotterdam. Dutch Patent 92.828 (issued December 15).

[3] P. Slump 1967 unpublished data.

[4] Thanks are due to Dr. Z. Bohak, Weizmann Institute of Science, Rehovoth, Israel, for a gift of pure lysinoalanine.

to rats for periods up to 13 weeks, and examination of the animals by clinical and pathological methods.

MATERIALS AND METHODS

Standard alkali treatment. An amount of material equivalent to 400 g crude protein was suspended in 3 liters 0.2 M sodium hydroxide. The pH was adjusted at 12.2 and the suspension kept at 40° for 4 hours. Thereafter, the slurry was acidified to pH 4.5 with 6 N HCl and centrifuged at 2700 rpm for 5 minutes; the residue was frozen (−20°) until used.

Amino acid analysis. Duplicate samples of 200 to 300 mg were hydrolyzed under reflux with 200 ml 6 N HCl for 22 hours. The acid was removed at reduced pressure in a rotary evaporator at 45°. The residue was dissolved in 0.2 N sodium citrate buffer, pH 2.2. In this hydrolysate all amino acids were determined except cystine, methionine and tryptophan. The S-amino acids were analyzed after oxidation with performic acid as described by Moore (9). Tryptophan was determined after autoclaving 1-g samples with 8 g $Ba(OH)_2 \cdot 8H_2O$ and 16 ml water for 8 hours at 120°, according to Slump and Schreuder (10).

The hydrolysates were chromatographed with the CIVO-automatic analyzer using the ion exchange resins Aminex A4 [5] for acid and neutral amino acids, Q15S [6] for basic amino acids and Sephadex G-25 [7] for tryptophan. The correction factors for destruction and incomplete hydrolysis were 1.05, 1.10, 1.07 and 1.08 for threonine, serine, isoleucine and valine, respectively. Upon chromatography with Q15S, lysinoalanine (LAL) appeared before lysine. Since this position is not specific for LAL, some chromatograms were developed with a PA35 [8] column for separating basic amino acids in physiological fluids. In these chromatograms LAL appears between ammonia and lysine.

In vitro digestibility. Pepsin digestibility was examined by incubating 2-g samples with 425 ml 0.1 N HCl and 100 mg pepsin powder [9] at 38 to 40°. Eight milliliters 0.1 N HCl were added after 16, 24 and 40 hours. After 48 hours the digest was cooled, brought to 500 ml and filtered. The total nitrogen content of the filtrate was determined by the Kjeldahl method.

Pepsin-pancreatin [10] digestibility of individual amino acids was determined as described by Akeson and Stahmann (11). The digests were deproteinized with 10% trichloroacetic acid, filtered and the filtrates analyzed for amino acids as described above. The amino acids released from the test protein were calculated after correction for the amino acids found in the blanks.

The rate of intestinal absorption of amino acids from enzymatic digests was examined in vitro by the everted sac technique of Wilson and Wiseman (12) using the small intestine distal to the duodenum of four adult female rats (200 g) which had been fasted for 20 hours. Three everted sacs (12 cm) were made from each intestine, filled with 2 ml Krebs-Henseleit solution (13) and incubated in pepsin-pancreatin digests of alkali treated protein, untreated protein and enzyme blanks with the protein omitted, respectively. After incubation at 37° for 45 minutes the fluids inside the four sacs in each of the three media were pooled and analyzed for amino acids.

Biological studies with rats. All feeding studies were carried out with weanling albino rats from the CIVO-colony (Wistar-derived) which were caged in groups of four to five rats in stainless steel wire-screen cages with raised-screen bottoms. Food and tap water were available at all times. Individual body weights were recorded every week.

Protein quality. Evaluation was carried out by the determination of net protein utilization (NPU) and true digestibility (D) according to the carcass-water method of Miller and Bender (14). The samples to be tested were incorporated into experimental diets as the sole source of protein to supply a protein level of 10% of the air-dried matter. The diets were made up to contain (in percent): sucrose, 30; minerals (15), 4; cellulose, 4; B-vitamin mixture (table 1), 2.2; vitamin ADE-preparation (table 1), 0.4; soybean oil, 5; and wheat starch to total 100.

[5] Bio-Rad Laboratories, Richmond, Calif., bulletin no. 115 A4, 1966.
[6] See footnote 5.
[7] Pharmacia, Uppsala, Sweden.
[8] Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif., bulletin no. A-TB-009A.
[9] Orthana A. S., Kemisk Fabrik, Kastrup, Denmark, 1:10 000 BPC.
[10] Hog pancreas powder, N.V. Organon, Oss, The Netherlands, activity 5 × NF.

After mixing the wet protein residues into the diets excess moisture was evaporated in a fluid bed dryer at 50° for 1 to 2 hours. Each series of diets included one protein-free diet. The diets were fed to three groups of two male and two female rats which had been fed the stock diet for 7 days after weaning. Food intake was recorded. The total amount of feces produced by all rats on each diet was collected on a wire screen at a distance of 2.5 cm underneath the screen bottoms of the cages. After a feeding period of 10 days the animals were killed; they were weighed, opened and dried to constant weight at 105° for 3 to 4 days. Total body nitrogen per group of four rats was calculated from the water content by means of a factor, expressing the relationship between body water and body nitrogen, which had been determined previously with rats of our colony. Total fecal nitrogen was determined by duplicate Kjeldahl analysis after drying and weighing.

Safety evaluation. To examine possible harmful effects of alkali-treated proteins, feeding studies were performed with soybean meal, casein and isolated soy protein (ISP) after standard alkali treatment. The residues obtained after neutralizing and centrifuging contained 87, 90 and 84%, respectively, of the protein in the starting material. The treated materials were incorporated into basal diets (table 1) to give the same level of protein intake by replacing 30% soybean meal, 15% casein or 20% ISP, respectively, by the corresponding amounts of the treated materials. The wet diets were dehydrated in a fluid bed dryer at 50° for 1 to 2 hours.

Groups of newly weaned rats were fed these diets for periods of 4, 6 or 13 weeks. Examinations of rats in the short-term tests were restricted to livers and kidneys, which were weighed and examined microscopically for histological changes. More extensive observations were made in the subchronic study. At week 13 hematological data were collected, consisting of hemoglobin content, packed cell volume, counts of red blood cells and of total and differential white blood cells. Also at week 13, urine analyses were made which comprised semiquantitative estimations, with sensitized paper, of pH, sugar, protein, occult blood and acetone, and microscopic studies

TABLE 1

Composition of basal diets used in feeding studies

	Diet		
	1	2	3
	%	%	%
Casein	10	25	10
Soybean oil meal	30		
Isolated soy protein [1]			20
Wheat starch	46.2	61.2	56.2
DL-Methionine	0.2	0.2	0.2
Vitamin B mixture [2]	1.2	1.2	1.2
Vitamin ADE-preparation [3]	0.4	0.4	0.4
Cellulose [4]	2	2	2
Minerals (15)	5	5	5
Soybean oil	5	5	5
By analysis:			
Calcium	0.82	0.81	0.79
Phosphorus	0.74	0.66	0.66

[1] Promine D, isolated soy protein, Central Soya Company, Chicago, Ill.
[2] In milligrams per 100 g diet: thiamine·HCl, 0.4; riboflavin, 0.5; pyridoxine·HCl, 0.25; niacin, 2.5; Ca pantothenate, 1.5; biotin, 0.01; folic acid, 0.1; vitamin B_{12}, 0.0025; choline chloride, 100; and sucrose, 1095.
[3] Per 100 g diet: 900 IU vitamin A; 300 IU vitamin D; and 10 mg vitamin E.
[4] Aku floc, AKU, Arnhem.

of the sediment. Possible kidney damage was determined by urinary examination of glutamic oxaloacetic transaminase activity (16) and of phenol red excretion (17) during 1 hour. Thereafter the rats were autopsied, examined grossly and the weights of 10 different organs of each rat were recorded. Tissue samples of these and of a variety of other organs were fixed in a neutral phosphate buffered 4% aqueous solution of formaldehyde, embedded in paraffin, sectioned (5 μ), stained with hematoxylin and eosin and examined under light microscopy.

RESULTS

Contents of lysine and lysinoalanine (LAL), and nutritive value of alkali-treated high protein materials. Table 2 contains data of some alkali-treated products partly obtained as such commercially, partly subjected to standard alkali treatment in the laboratory. Each of the laboratory-treated products contained appreciable amounts of LAL except for coconut meal. A decrease in lysine content and NPU by alkali treatment was noticeable as far as control sam-

TABLE 2

Effects of alkali treatment on protein quality and contents of lysine and lysinoalanine (LAL) in some high protein products

Product examined	Nutritive value		Lysine	LAL
	NPU	D		
			g/16 g N	*g/16 g N*
Soybean oil meal [1]	63	89	6.2	0.0
Treated with water, pH 7	61	91	6.8	0.0
Alkali treated [2]	41	91	6.1	0.57
Alkali treated, protein isolate	24	92	5.4	0.80
Casein [3]	63	101	8.3	0.0
Alkali treated	53	90	7.7	1.15
Sodium caseinate [4]	62	98	8.8	0.0
Confectionery product, vegetable [5]	40	94	1.4	0.0
Confectionery product, animal [5]	3	49	4.8	5.5
Animal protein concentrate, pH 12.2, 50°	61	85	9.1	1.1
pH 12.2, 65°	61	—	8.7	1.0
pH 12.2, 80°	26	79	8.1	2.3
Groundnut meal,[1] alkali treated	—	—	3.0	0.3
Coconut meal,[1] alkali treated	—	—	2.5	0.0
Sesame protein,[6] alkali treated	—	—	3.1	1.1
Brewer's yeast protein,[6] alkali treated	—	—	6.5	0.8

[1] Defatted commercial product as used in feeds for farm animals.
[2] Alkali treated means treated under standard conditions at pH 12.2, at 40° for 4 hours.
[3] Acid-precipitated commercial product.
[4] Solubilized product obtained by converting acid-precipitated casein into its sodium salt.
[5] Commercial foaming agent in confectionery.
[6] Proteins from commercial feed product isolated by dissolving at pH 7.5, centrifuging and subsequently treating the supernatant at pH 12.2 and 40° for 4 hours.

ples were tested. The protein isolated from the alkali-soluble part of soybean meal by acidifying and centrifuging contained more LAL and less lysine, and had a lower NPU than the alkali-treated, not separated, soybean oil meal. In contrast to casein after standard alkali treatment, no LAL and no decreased NPU was shown by commercial sodium caseinate.

Digestibility was distinctly decreased in laboratory-treated casein but not in soybean oil meal treated in the same way. A commercial confectionary product of animal origin contained a large amount of LAL and showed very poor utilization. Experimental samples of an animal protein concentrate treated at pH 12 and increasing temperature contained LAL, and showed a decrease in lysine content and nutritive value when treated at 80°.

From these results it appeared that drastic treatment of food proteins with alkali may result in the formation of LAL which is attended with decreased lysine contents and impaired nutritive value.

Fecal analyses showed that LAL is poorly absorbed. Upon ingesting the compound with diets containing either alkali-treated soybean meal, ISP or casein the excretion in the feces of rats amounted to 38, 39 and 65%, respectively. Only 1.5% of the LAL ingested was recovered in the urine of rats fed alkali-treated ISP. The total amount of LAL recovered in feces and urine in the latter experiment was 40%. The fate of the remaining 60% was not known.

Effects of varying pH, temperature and duration of treatment on amino acid composition and nutritive value of isolated soy protein (ISP). The effects of different conditions of alkali treatment on amino acid composition and nutritive quality were studied with ISP. The pH was varied between 7 and 12.2, the temperature between 23 and 80°, and the duration of treatment from 1 to 8 hours. The determinations were carried out only with that part of the starting material which was recovered by centrifuging at pH 4.5. The amounts of material recovered decreased with increasing

severity of treatment and ranged from 95 to 45%.

Results are presented in table 3. Sample 5 was included three times, to complete three different treatment series, although its amino acid composition was determined only once.

A number of amino acids, namely methionine, threonine, arginine, histidine, glutamic acid, proline and cysteic acid, occurred at relatively constant levels after all treatment conditions. A comparable number of other amino acids, namely isoleucine, leucine, tyrosine, phenylalanine, valine, alanine, aspartic acid and glycine, showed increased contents in the drastically treated samples 5, 6, 9 and 10. The contents of only three amino acids, i.e., lysine, cystine and serine, were distinctly decreased by certain treatments with alkali. Cystine was decreased about 50% or more in all samples treated at pH 12.2. The extent of cystine destruction at pH 12.2 and 40° was independent of the duration of the treatment between 1 and 8 hours, but increased when the temperature was raised above 40°. The contents of lysine and serine were distinctly decreased only after exposure to alkali at temperatures above 40° (samples 8 and 9). Obviously cystine is the amino acid which is most sensitive to alkali.

Lysinoalanine was present in all samples treated at pH 12.2 and also in the sample treated at pH 10. The amount of LAL found at pH 12.2 increased with increasing duration of alkali treatment and also with increasing temperature.

Net protein utilization was considerably reduced in all samples treated at pH 12.2 as could be expected from the lower contents of S-amino acids. The effect became more pronounced with increased exposure time and temperature. Alkali treatment also reduced true digestibility (D). The effect was enhanced by both increased duration and temperature. Very low D values were obtained with protein exposed to pH 12.2 at 60 and 80°.

A distinct inverse relationship between LAL and NPU is noticeable from the figures of table 3. Calculation of the correlation coefficient resulted in a value of $r = -0.96$.

Effect of amino acid supplementation of isolated soy protein (ISP) before and after alkali treatment. To examine how far the lowered NPU of alkali-treated ISP could be restored to normal by supplementing the protein with amino acids the effects of various supplements were determined by assays of NPU and D. The results are presented in table 4.

The addition of increasing levels of methionine resulted in a considerably improved NPU of both untreated and treated ISP. The maximum value obtained with the treated material, however, remained far below that of the untreated ISP. The difference may be explained by the lower digestibility of the treated material. In a second experiment, lysine together with methionine did not bring about a further rise of NPU over that of methionine alone in the first series, which indicated that lysine was not limiting the nutritive value of ISP either before or after alkali treatment. Threonine, however, caused a distinct further improvement of both untreated and treated ISP, which showed that threonine was the second limiting amino acid.

In vitro release and absorption of amino acids from untreated and alkali-treated isolated soy protein (ISP). Amino acid analyses of pepsin-pancreatin digests of untreated and alkali-treated ISP revealed (table 5) that the enzymatic release of several amino acids such as valine, arginine, alanine, aspartic and glutamic acids, and glycine was similar in both samples. Practically no proline was released in either digest. The other amino acids in alkali-treated ISP were distinctly less susceptible to enzymatic release than those in untreated ISP. Despite this difference the pepsin digestibility was the same in both samples, i.e., 98%. This discrepancy is ascribed to the fact that the pepsin digestibility method measures solubility of proteins rather than actual digestibility.

Cystine, cysteic acid, tryptophan and LAL were not determined in these digests because of low levels and difficulties in chromatographic separation.

Marked differences between the enzymatic digests of untreated and treated ISP were observed when the absorption of individual amino acids through the intestinal wall was examined in vitro. The results are presented in table 5, expressed as percentage of the total amount of amino acids ab-

TABLE 3

Amino acid composition and nutritive value after various treatments of isolated soy protein (ISP)

Sample no.	1	2	3	4	5	6	7	5	8	9	10	11	12	5
Treatment														
pH	–	7	12.2	12.2	12.2	12.2	12.2	12.2	12.2	12.2	7	8	10	12.2
Temperature, °C	–	23	40	40	40	40	23	40	60	80	40	40	40	40
Duration, hr	–	0	1	2	4	8	4	4	4	4	4	4	4	4
							g/16 g nitrogen							
Isoleucine	5.0	5.0	5.0	5.3	5.3	5.6	4.8	5.3	5.3	5.7	5.0	5.2	5.1	5.3
Leucine	7.5	7.7	7.8	8.3	8.4	8.7	8.1	8.4	9.1	9.7	8.1	8.0	7.9	8.4
Lysine	5.7	5.8	5.6	5.2	5.4	5.1	5.6	5.4	4.9	4.7	5.8	5.5	5.7	5.4
Tyrosine	3.6	3.7	3.8	4.0	3.9	4.2	3.7	3.9	4.3	4.5	3.8	3.9	3.6	3.9
Phenylalanine	5.3	5.4	5.6	5.8	5.7	6.0	5.6	5.7	6.3	6.4	5.6	5.6	5.3	5.7
Cystine	0.87	0.81	0.40	0.40	0.37	0.40	0.47	0.37	0.32	0.25	0.83	0.83	0.80	0.37
Methionine	1.25	1.15	1.16	1.17	1.16	1.23	1.17	1.16	1.25	1.39	1.26	1.26	1.27	1.16
Threonine	3.5	3.5	3.5	3.5	3.6	3.7	3.6	3.6	3.5	3.4	3.8	3.9	3.8	3.6
Tryptophan		1.1			1.1	1.15		1.1		1.3				1.1
Valine	5.0	5.0	5.0	5.0	5.5	5.6	5.1	5.5	5.6	6.4	5.2	5.1	5.2	5.5
Arginine	7.7	7.7	8.0	8.0	7.9	7.7	7.9	7.9	7.9	7.5	8.0	7.7	7.7	7.9
Histidine	2.3	2.3	2.4	2.4	2.4	2.3	2.3	2.4	2.4	2.4	2.4	2.4	2.3	2.4
Alanine	3.7	3.7	3.8	3.8	4.0	4.3	3.8	4.0	4.4	4.6	3.8	3.9	3.7	4.0
Aspartic acid	11.7	11.9	12.0	12.0	12.5	12.8	12.3	12.5	12.7	12.9	11.3	12.1	11.5	12.5
Glutamic acid	20.9	20.6	20.4	20.5	20.7	20.8	21.4	20.7	20.1	19.5	21.3	21.4	22.0	20.7
Glycine	4.0	4.0	4.1	4.1	4.2	4.3	4.1	4.2	4.4	4.6	4.1	4.1	3.9	4.2
Proline	5.0	4.8	4.9	4.9	4.9	5.1	5.0	4.9	5.0	4.9	5.0	5.1	5.2	4.9
Serine	5.6	5.6	5.5	5.5	5.8	5.7	5.8	5.8	5.0	5.0	5.8	5.9	5.8	5.8
Cysteic acid	0.25	0.27	0.24	0.24	0.26	0.25	0.23	0.26	0.26	0.25	0.27	0.27	0.26	0.26
Lysinoalanine	0.0	0.0	0.42	0.68	0.83	1.09	0.40	0.83	1.71	2.08	0.0	0.0	0.10	0.83
NPU	35	35	19	12	15	−2	19	1	−3	−14	34	38	36	11
D	97	97	95	92	90	86	94	93	76	70	98	95	96	96

TABLE 4

Effect of amino acid supplementation on nutritive value of isolated soy protein (ISP) before and after standard alkali treatment

Protein and supplements	Nutritive value		
	NPU	D	BV
ISP	39	95	41
+L-methionine 0.1%	55	94	59
+L-methionine 0.2%	66	96	69
+L-methionine 0.4%	64	96	67
ISP, alkali treated	2	86	2
+L-methionine 0.1%	32	86	37
+L-methionine 0.2%	39	86	45
+L-methionine 0.4%	33	89	38
ISP	32	99	33
+L-methionine 0.3% +L-lysine 0.3%	64	99	64
+L-methionine 0.3% +L-lysine 0.3% +L-threonine 0.2%	77	100	77
ISP, alkali treated	10	91	11
+L-methionine 0.3% +L-lysine 0.3%	40	91	39
+L-methionine 0.3% +L-lysine 0.3% +L-threonine 0.2%	62	93	67

TABLE 5

Amino acid release in vitro by pepsin-pancreatin from untreated and alkali-treated isolated soy protein (ISP) and amino acid absorption from the digest into everted intestinal sacs

Amino acid	Released by pepsin-pancreatin		Absorbed into intestinal sacs	
	Control	Treated	Control	Treated
	g/16 g N	*g/16 g N*	*g/100 g amino acid*	*g/100 g amino acid*
Isoleucine	1.05	0.96	6.3	8.2
Leucine	3.74	3.56	10.4	12.7
Lysine	2.28	2.10	7.0	8.4
Tyrosine	2.30	2.02	4.5	5.5
Phenylalanine	3.32	2.70	7.0	8.4
Methionine + methionine sulfoxide	0.18	0.10	1.3	0.8
Threonine	0.35	0.26	4.4	3.1
Valine	1.1	1.0	6.9	8.2
Arginine	3.9	3.9	9.1	11.9
Histidine	0.53	0.41	2.8	2.7
Alanine	0.61	0.59	6.1	6.3
Aspartic acid	0.06	0.07	8.6	4.9
Glutamic acid	0.33	0.35	12.0	9.3
Glycine	0.12	0.14	2.9	1.4
Proline	0.0	0.0	4.4	4.4
Serine	0.35	0.25	6.5	3.7
Total	20.2	18.4	100	100

sorbed after correction for the blanks. Most of the nonessential amino acids were absorbed from the digest of untreated ISP rather than from the digest of alkali-treated ISP. The essential amino acids, however, were in general better absorbed from treated ISP, except for methionine and threonine.

Short-term feeding trials. Table 6 summarizes results of three successive experiments with diets containing relatively high levels of either soybean oil meal, casein or ISP treated with alkali under standard conditions (pH 12.2, 40°, 4 hours). Each of the test and control diets contained 10% untreated casein and a supplement of 0.2% *dl*-methionine (table 1). Experimental periods were 4 to 6 weeks.

Gain in body weight was not significantly affected in any of the experiments. The

TABLE 6

Gain in body weight, relative weight of liver and kidney and degree of nephrocalcinosis upon feeding different proteins with and without alkali treatment

Alkali-treated protein in the diet	Males				Females			
	Gain in body wt	Liver	Kidney	Nephrocalcinosis	Gain in body wt	Liver	Kidney	Nephrocalcinosis
	g/rat	*% body wt*	*% body wt*	[1]	*g/rat*	*% body wt*	*% body wt*	[1]
Groups of 10 males and 10 females for 6 weeks (exp. 1)								
None (diet 1, table 1)	146.2	3.72	0.81	±	87.6	3.58	0.74	+
Soybean meal	150.2	3.78	0.82	+	94.8	3.52	0.81	+++
Groups of 10 males and 10 females for 4 weeks (exp. 2)								
None (diet 2, table1)	114.8	4.65	0.87	±	76.6	4.16	0.89	++
Casein, HCl precipitated	115.4	4.84	0.90	±	79.9	4.19	0.91	++
Casein, acetic acid precipitated	115.7	4.93 [2]	0.93	±	82.7	4.56 [3]	0.96	+++
Groups of 5 females for 6 weeks (exp. 3)								
None (diet 3, table 1)					110.3	3.73	0.83	+
ISP					102.5	3.93	0.87	++
ISP + $CaCl_2 \cdot 2H_2O$					99.3	3.91	0.84	±

[1] Grading system: ± minimal; + slight; ++ moderate; +++ severe.
[2] Significantly different from the control at a 5% level.
[3] Significantly different from the control at a 1% level.

weight of liver and kidney was generally slightly higher on the diet with treated protein than on the control diet. Microscopically no differences were noticeable between the livers of treated and control rats in any of these studies. In experiment 1 the kidneys of female test rats showed distinct changes consisting of heavy calcareous deposits in the cortico-medullary region attended with distorted tubules. Similar renal changes were present also in the controls, though less severe. This phenomenon, nephrocalcinosis, is a common observation in the strain of rats used and occurs mainly in females. It is known to be aggravated in rats fed diets either low in calcium, high in phosphorus or low in magnesium (18, 19). The levels of these minerals, however, were adequate and similar in all diets, basal as well as experimental diets, and amounted to 0.8, 0.7 and 0.05%, respectively.

To examine whether the acid used for neutralizing and precipitating the protein might affect nephrocalcinosis experiment 2 was carried out with alkali-treated casein precipitated with either hydrochloric or acetic acid. The kidneys again showed varying degrees of nephrocalcinosis in all groups. The phenomenon was more pronounced in females on acetic acid-precipitated casein than in other groups. This difference, together with the absence of increased nephrocalcinosis on the diet with HCl-precipitated casein, and the distinct nephrocalcinosis on treated soybean meal precipitated with the same acid, does not suggest that the kind of acid used affected the renal changes.

Severe nephrocalcinosis induced by high levels of dietary phosphorus is counteracted by increasing the level of dietary calcium (19). Therefore, the effect of a supplement of 1% $CaCl_2 \cdot 2H_2O$ was examined with a diet containing alkali-treated ISP. The results again showed increased renal changes in rats fed the diet containing the alkali-treated protein, but when fed the same diet supplemented with calcium the female rats showed a remarkably low degree of renal calcinosis, even less than the controls. These results suggest that the renal changes on diets with alkali-treated proteins are related with mineral metabolism.

Subchronic feeding study. Average results obtained on diets with alkali-treated 20% ISP or untreated (table 1) for a 13-week feeding period are presented in table 7. Body weights on treated protein were lower than in controls though not significantly ($P > 0.05$). Examination of blood and urine failed to show any significant differences between the two groups. Microscopy of the urine sediment and differential counts of white blood cells, the results of which are not included in the table, were very similar. Organ weights and gross autopsy findings did not show treatment-related differences. Microscopic examination of about 30 different organs and tissues did not reveal any distinct abnormalities, except for the kidney of one treated female in which severe nephrocalcinosis was observed. In the remaining four treated females and in the control females the phenomenon was rated minimal or slight. From the results of the feeding studies with alkali-treated proteins it appears that the only finding of possible toxicological significance was an increased degree of renal calcinosis in females which could be alleviated by a dietary supplement of calcium.

DISCUSSION

These studies have shown that drastic treatment of food proteins with alkali, at pH 12.2 and 40° for 4 hours, may induce chemical changes which are attended with the occurrence of a new amino acid, lysinoalanine (LAL), and with decreased contents of cystine and, to a lesser extent, lysine. More drastic treatment at a temperature between 40 and 80° also destroys serine and arginine. Destruction of cystine and lysine in alkali-treated fish meal has been reported in the literature (20).

A primary change in proteins by alkali treatment is supposed to be the formation of dehydroalanine residues from cystine and serine residues (5). This compound may react with the ε-amino group of lysine, which results in the formation of LAL, or with intact cystine residues, which leads to the formation of lanthionine. The latter amino acid was not observed in our chromatograms, but small amounts may have escaped detection.

Destruction of cystine or lysine in proteins often means decreased nutritive value

TABLE 7

Average results of growth, examination of blood and urine and organ weights after feeding isolated soy protein (ISP) before and after alkali treatment to groups of five male and five female rats for 13 weeks

Criteria	Untreated		Treated	
	Males	Females	Males	Females
Body wt, g/rat	318	202	304	174
Urine				
Percentage phenol red excretion in 1 hr	50.4	64.1	51.7	67.3
UGOT, RF units	5.8	15.2	8.3	18.3
pH	6	6	6	6
Sugar	0	0	0	0
Protein	++	++	++	++
Occult blood	0	0	0	0
Acetone	0	0	0	0
Blood				
Hemoglobin, g/100 ml	15.8	16.6	15.7	17.6
Hematocrit, %	49.4	49.5	49.5	51.1
Red blood cells, 10^{-6}/mm^3	8.5	8.3	8.8	8.7
White blood cells, 10^{-3}/mm^3	17.2	14.7	20.0	15.9
Organ weights, % body wt				
Heart	0.346	0.380	0.346	0.410
Kidney	0.86	0.66	0.81	0.72
Liver	2.92	2.80	3.01	2.74
Spleen	0.177	0.205	0.194	0.229
Brain	0.56	0.82	0.57	0.92
Testicle/ovary	0.95	0.031	0.89	0.035
Thymus	0.100	0.158	0.103	0.174
Pituitary	0.0033	0.0050	0.0033	0.0056
Thyroid	0.0059	0.0084	0.0062	0.0096
Adrenal	0.0131	0.0220	0.0128	0.0246
Renal calcinosis				
Negative	0/5	0/5	1/5	0/5
Minimal	3/5	1/5	4/5	1/5
Slight	2/5	4/5	0/5	3/5
Moderate	0/5	0/5	0/5	0/5
Severe	0/5	0/5	0/5	1/5

because the majority of food proteins is limited by either the sulfur-containing amino acids or lysine. Exposure of ISP to pH 12.2 in the present experiment was invariably accompanied by a decrease in NPU. The extent of impairment of protein quality was distinctly correlated with the severity of alkali treatment.

The NPU of ISP samples submitted to alkali treatment varying in pH, temperature and duration showed a highly significant negative correlation with the LAL contents of the samples. Therefore, the LAL content of proteins is a good criterion for measuring damage to proteins caused by alkali, as is the available lysine content for estimating damage caused by heat treatment. A decrease in cystine is also a good indicator of alkali damage, although LAL is more sensitive and more specific.

At pH 12.2, protein damage as shown by reduced cystine content, presence of LAL and lowered NPU was apparent when applied at 40° for 1 hour, and at room temperature for 4 hours. Treatment of ISP at pH 12.2 and room temperature for 60 and 10 minutes resulted in LAL contents of 0.2 and 0.03 g/16 g N. After 3 hours at pH 9 and 90° LAL was not detectable.

The results obtained suggest that exposure of ISP at pH 12.2, even at room temperature for a relatively short time, will destroy some of the cystine, which inevitably results in decreased nutritive value.

Bressani et al. (21) did not obtain a significant difference in NPU or PER between ISP before and after exposure to alkali of pH 12.2 at room temperature for less than 10 minutes.

The NPU assays of alkali-treated ISP supplemented with amino acids showed methionine to be the first limiting amino acid and threonine the second. The utilization of the methionine-supplemented protein treated with alkali was considerably lower than that of the untreated protein with respect to both the total protein consumed and the digestible part as well. Since the amounts of threonine in treated and untreated proteins were similar, the difference in nutritive value after methionine supplementation suggests a decreased utilization of threonine in the treated protein. This agreed with a decreased rate of threonine absorption from alkali-treated ISP observed in vitro. Decreased utilization of threonine might be caused by alkaline racemization of amino acids in intact proteins (22, 23) leading to the formation of *d*-threonine which is not utilized by rats (24).

Some authors reported toxic effects in rats (25) and chickens (26) from short-time feeding of casein or fish meal severely treated with alkali. The present feeding studies with less severely treated proteins failed to show any detrimental effect apart from an increased degree of nephrocalcinosis in females which was prevented by additional dietary calcium. The mechanism of this phenomenon, which apparently is related with calcium-phosphor metabolism, remains to be elucidated.

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LITERATURE CITED

1. Sullivan, J. P. 1943 Protein concentrates from grasses. Science, *98:* 363.
2. Circle, S. J., and D. W. Johnson 1958 Edible isolated protein. In: Processed Plant Protein Foodstuffs, ed., A. M. Altschul. Academic Press, New York, p. 403.
3. Screenivasamurthy, V. 1967 Detoxification of aflatoxin in peanut meal by H_2O_2. J. Assoc. Offic. Agr. Chem., *50:* 350.
4. Soy fibers; a new approach to vegetable protein acceptability. 1967 Nutr. Rev., *25:* 305.
5. Patchornik, A., and M. Sokolovsky 1964 Chemical interactions between lysine and dehydroalanine in modified bovine pancreatic ribonuclease. J. Amer. Chem. Soc., *86:* 1860.
6. Bohak, Z. 1964 N_ε-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem., *239:* 2878.
7. Horn, M. J., D. B. Jones and S. J. Ringel 1941 Isolation of a new sulfur-containing amino acid (lanthionine) from sodium carbonate-treated wool. J. Biol. Chem., *138:* 141.
8. Ziegler, K., I. Melchert and C. Lürken 1967 N^δ-(2-amino-2-carboxyethyl)-ornithine, a new amino acid from alkali-treated proteins. Nature, *214:* 404.
9. Moore, S. 1963 On the determination of cystine as cysteic acid. J. Biol. Chem., *238:* 235.
10. Slump, P., and H. A. W. Schreuder 1969 Determination of tryptophan in foods. Anal. Biochem., *27:* 182.
11. Akeson, W. R., and M. A. Stahmann 1964 A pepsin pancreatin digest index of protein quality evaluation. J. Nutr., *83:* 257.
12. Wilson, T. H., and G. Wiseman 1954 The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. (London), *123:* 116.
13. Krebs, H. A., and K. Henseleit 1932 Untersuchungen über die Harnstoffbildung im Tierkörper. Z. Physiol. Chem., *210:* 33.
14. Miller, D. S., and A. E. Bender 1955 The determination of the net utilization of proteins by a shortened method. Brit. J. Nutr., *9:* 382.
15. 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.
16. Reitman, S., and S. Frankel 1957 A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Amer. J. Clin. Pathol., *28:* 56.
17. Sharratt, M., and A. C. Frazer 1963 The sensitivity of function tests in detecting renal damage in the rat. Toxicol. Appl. Pharmacol., *5:* 36.
18. MacKay, E. M., and J. Oliver 1934 Renal damage following the ingestion of a diet containing an excess of inorganic phosphate. J. Exp. Med., *61:* 319.
19. Forbes, R. M. 1963 Mineral utilization in the rat. I. Effects of varying dietary ratios of calcium, magnesium and phosphorus. J. Nutr., *80:* 321.
20. Carpenter, K. J., J. Duckworth, G. M. Ellinger and D. H. Shrimpton 1952 The nutritional evaluation of protein concentrates obtained from the alkali digestion of herrings. J. Sci. Food Agr., *3:* 278.
21. Bressani, R., F. Viteri, L. G. Elías, S. de Zaghi, J. Alvarado and A. D. Odell 1967

Protein quality of a soybean protein textured food in experimental animals and children. J. Nutr., *93:* 349.

22. Pollock, G. E., and L. H. Frommhagen 1968 The extent of racemization of some amino acids in dilute alkali-treated protein and soil humic and fulvic acid. Anal. Biochem., *24:* 18.
23. Geschwind, I. I., and C. H. Li 1964 Effects of alkali-heat treatment on β-melanocyte stimulating hormone. Arch. Biochem. Biophys., *106:* 200.
24. West, H., and H. E. Carter 1938 Synthesis of α-amino-β-hydroxy-*n*-butyric acids. VI. Preparation of *d*- and *l*-allothreonine and nutritive value of the four isomers. J. Biol. Chem., *122:* 611.
25. Ramasarma, G. B., L. M. Henderson and C. A. Elvehjem 1949 Purified amino acids as a source of nitrogen for the growing rat. J. Nutr., *38:* 177.
26. Carpenter, K. J., and J. Duckworth 1950 The nutritive value of herring "alkali-reduction" meal for chicks. J. Agr. Sci., *40:* 44.

Effect of Copper Deficiency on Chick Bone Collagen and Selected Bone Enzymes [1,2,3]

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ABSTRACT Studies were conducted with chicks to determine the effect of copper deprivation on collagen formation, mineral content and activity of selected enzymes in bone tissue. After 7 to 10 days on copper-deficient diets, collagen extracted from copper-deficient chick bone contained less aldehyde and was more easily solubilized than collagen from control bone. Collagens were extracted with acetic acid, guanidine·HCl, NaCl and alkali. In all cases, copper-deficient bones yielded greater amounts of soluble collagen. Bone enzyme studies indicated that amine oxidase activity was reduced 30 to 40% in deficient chicks as compared with controls. In the enzyme assays for amine oxidase the in vitro addition of copper resulted in an elevation of activity to that of controls. Bone cytochrome oxidase was also markedly reduced with copper deficiency. The copper-containing enzymes in bone appear to be similar to those reported in other tissues. Catalase was not affected by the deficiency. Bone mineral analyses demonstrated that the calcium, phosphorus and magnesium content of bone ash was normal in both experimental groups. The copper content of control bone was twice that of deficient bone. Extraction of mineral from bone of both copper-deficient and control chicks removed over 96% of the bone mineral, but much of the bone copper was not extracted and appeared to be tenaciously bound to the organic phase of bone.

Copper deficiency results in disorders which affect the integrity of connective and elastic tissues. In the chick and turkey poult, these anomalies are characterized by aortic rupture (1–3) and fragile, deformed bone (4, 5). In vascular tissue the disorder is related to a decrease in the intramolecular cross-linking of elastin (6).

Collagen has also been shown to contain intramolecular cross-links (7, 8). The exact nature of the cross-links in collagen is not known, but the biosynthetic sequence for the formation of collagen cross-links appears to be similar to that of elastin. This sequence presumably involves the oxidative deamination of the ε-amino group of lysine with subsequent condensation with other aldehydic or amine groups (8–10).

The solubility of collagen is inversely related to the degree of cross-linking (7). It has been demonstrated that collagen from copper-deficient aortic tissue and tendon is more soluble than collagen from corresponding controls (4, 11). In this report, the effects of copper deprivation on bone collagen and several enzymes are studied in an attempt to further clarify the apparent interrelationship of copper and collagen to bone disorders.

MATERIALS AND METHODS

Chicks. Groups of 1-day-old Hubbard White Mountain cockerels were fed copper-deficient (< 1 ppm) and copper-supplemented (25 ppm) diets [5] consisting primarily of skim milk powder (55%), glucose (35%) and corn oil (5%). The chicks received deionized water ad libitum and were housed in epoxy-coated electrically heated brooders. Weight gain and the percentage of aortic elastin after alkali extraction (12) were periodically determined to estimate the degree of deficiency.

Preparation of bone homogenates. At the termination of the experimental pe-

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[5] General Biochemicals, Inc., Chagrin Falls, Ohio, catalog no. 170210.

riods, chicks from the two groups were killed and the right femurs removed. The bones were cleaned of adhering tissue, broken and the marrow removed by washing with cold saline using a needle and syringe. The cleaned fragments were then placed in cold 0.1 M sodium phosphate buffer (pH 7.5) containing 0.25 M sucrose; they were homogenized in a blender [6] at full speed for 2 to 3 minutes with the container immersed in ice water. The homogenates were diluted with buffer to give a 10% (w/v) solution and centrifuged for 30 minutes at 10,000 × *g* (4°). The 10,000 × *g* supernate and pellet were separated.

Enzyme studies. Bone amine oxidase was assayed by the method of Gorkin et al. (13) with slight modification. One milliliter of the 10,000 × *g* supernate from each femur and 2 ml of 0.1 M phosphate buffer (pH 7.5) containing 10 μmoles of benzylamine were incubated for 2 hours at 41° in a Dubnoff metabolic shaker.[7] At the termination of the incubation, the production of benzaldehyde was determined spectrophotometrically at 250 mμ using a spectrophotometer.[8] Absorbance at 250 mμ in all cases was proportional to both protein concentration and incubation time. Activity is expressed as μmoles benzaldehyde per hour per milligram protein. Benzylamine was chosen since it is generally reacted upon by most copper-containing monoamine oxidases (14).

Cytochrome oxidase activity was determined by the method of Copperstein and Lazarow (15). Bone homogenates were centrifuged at 700 × *g* to remove bone fragments and the supernatant fraction was centrifuged for 30 minutes at 10,000 × *g*. The resultant pellet (10,000 × *g*), presumed to consist primarily of mitochondria, was subjected to sonication (250 w, 10,000 cycles/second) for 2 to 3 minutes (5°). One-tenth milliliter of the sonicated fraction was added to 2.9 ml of reduced cytochrome *c* (1.7×10^{-5} M) in 0.03 M phosphate buffer (pH 7.3) at room temperature. The decrease in absorbance at 550 mμ was followed for 3 minutes. Activity is expressed as log [ferrocytochrome c] per minute per milligram protein.

Catalase activity in the 10,000 × *g* supernate was determined by the decrease in absorbance of H_2O_2 at 250 mμ (16). The substrate consisted of 1 ml Superoxal (Merck 30% H_2O_2) diluted in 500 ml of 0.05 M phosphate buffer (pH 7.0). The assay mixture contained 0.05 ml of the supernatant fraction and 1.95 ml of buffered H_2O_2 solution. The initial absorbance of the assay mixture versus a water plus supernatant fraction blank was 0.85, and the decrease in absorbance was measured at 20-second intervals for 2 minutes. The molar absorbancy index for H_2O_2 at 250 mμ is 43.6 in a 1-cm cuvette, and units were defined as △ A/minute × 1000 ÷ 43.6 × milligrams protein per milliliter reaction mixture.

Protein in all enzyme studies was determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Collagen extraction. Four methods were used to extract bone collagens. Chicks at 10 to 20 days of age were killed and the femurs removed. Femurs were cleansed of adhering tissue and freeze-dried; they were then ground with mortar and pestle and weighed. The ground bone from individual femurs was then placed in 0.1 M NaOH (100 mg/10 ml) for 12 hours at room temperature (18). After extraction, the solutions were centrifuged at 110,000 × *g* for 90 minutes in a preparatory ultracentrifuge.[9] The supernatant fraction was separated and the residue was again extracted for 2 hours with 0.1 M NaOH and recentrifuged. The 110,000 × *g* supernates were combined and the total protein was precipitated by addition of trichloroacetic acid (TCA) (20% w/v). The total collagen from the 110,000 × *g* residue and the precipitated protein from alkali extract was then extracted with 0.3 M TCA at 90° (19) and made 6 N with HCl. After hydrolysis for 12 hours in sealed tubes, the hydroxyproline content was determined (20). Collagen is expressed as milligrams hydroxyproline × 7.7/mg dried bone × 100.

Collagen (as gelatin) was also extracted from dried bone with 0.1 M NaCl (50 ml/g bone) at 60° for 72 hours (21). Solutions were clarified by centrifugation (30,000

[6] Ivan Sorval Inc., Type OM, Norwalk, Conn.
[7] Precision Scientific Company, Chicago, Ill.
[8] Beckman DU, Beckman Instruments, Inc., Fullerton, Calif.
[9] Model L2-50 ultracentrifuge; Spinco Division, Beckman Instruments. Inc., Palo Alto, Calif.

$\times g$ for 90 minutes) and the hydroxyproline content of the supernatant fraction was determined. Other bone samples were extracted similarly with 0.5 M acetic acid or 5 M guanidine·HCl for 3 weeks and 1 week, respectively. Extractions with acetic acid and guanidine were carried out at 4°, and in some cases bones were decalcified with 0.5 M EDTA prior to extraction. In extraction studies with NaCl, acetic acid and guanidine, only diaphyseal bone was used to avoid contamination with cartilaginous areas.

Aldehyde determination. The acetic acid soluble collagen was purified as described by Miller et al. (22) prior to the determination of the aldehyde content by the method of Paz et al. (23).

Mineral determinations. Bone samples were extracted with ethanol and diethyl ether and dried. The fat-free dried samples were then ashed (550° for 24 hours) and the percentage ash determined. The calcium and magnesium content of ash was determined with an atomic-absorption spectrophotometer.[10] The phosphorus content of ash was determined by the method of Fiske and Subbarow (24).

The left and right femurs from ten 14-day-old control and copper-deficient chicks were removed, cleaned, extracted and dried. The right femur was broken into several pieces and placed in 5% (w/v) aqueous EDTA and demineralized for 4 days. The solution was changed daily (50 ml EDTA solution/g bone) and agitated regularly. The corresponding left femur was used to determine the percentage ash and total bone copper. Copper was determined in the ash from the left femur and in the residue after EDTA extraction (right femur) by atomic-absorption spectrophotometry (25). The copper content of extracted residue is expressed as micrograms copper per gram ash from the corresponding left femur.

Liver copper was also analyzed by atomic-absorption spectrophotometry. Liver samples were first freeze-dried; then they were weighed and ashed (500° for 24 hours). The ash was dissolved in 0.5 N HCl and analyzed directly.

RESULTS AND DISCUSSION

As demonstrated by other workers (1–3), there was a reduction in chick weight and the percentage aortic elastin in the copper-deficient group (table 1). Liver and bone copper were also markedly reduced. Although the percentage bone ash showed only a slight reduction in copper-deficient chicks compared with controls, the femurs from the copper-deficient chicks were quite brittle and fragile. The calcium, magnesium and phosphorus content of ash was normal in both groups.

The interrelationship of dietary copper and amine oxidase activity in the formation of vascular and connective tissue has been established (11, 26). Copper deficiency results in decreased levels of this enzyme. Bone amine oxidase activity, as measured by benzylamine oxidation, was also reduced significantly in copper-deficient chicks within 7 days of feeding the diet compared with controls (fig. 1). At about this time there also appeared to be a slight decrease in the aldehyde content of collagen from copper-deficient bone (fig. 2). In another experiment a significant difference in aldehyde content could be clearly demonstrated at 20 days (table 2). The aldehyde content of collagen should be an indication of potential cross-linking, if the final steps of the process involve condensation or coupling of aldehydic functional groups (27).

A decrease in cross-linking should also bring about a concomitant decrease in insoluble collagen. This effect could be demonstrated by differences in the percentages of insoluble residue from diaphysis bone cylinders after extraction for 7 days with 5 M guanidine·HCl containing 5% EDTA (fig. 2). At 10 days feeding of the diets there was a significant decrease in the insoluble organic matter from bone of deficient chicks compared with controls.

Both the aldehyde content of soluble collagen and bone amine oxidase activity were greater in 1-day-old chicks than in chicks of 2 to 5 days of age. Since this was accompanied by a decrease in the amount of insoluble residue from bone of 2- to 5-day-old chicks, it was concluded that soluble and nonoxidized precursors of the bone organic matrix may be synthesized very rapidly at this time. The decrease in bone amine oxidase level at 3 days was pre-

[10] Model 214, Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Effect of copper deficiency on weight, aortic elastin, liver copper and the mineral content of bone ash

Diet	Age	Aortic elastin [1]	Avg wt [2]	Liver copper [3]	Bone ash [4]	Mineral content of ash			
						Copper [5]	Calcium [6]	Phosphorus [6]	Magnesium [6]
	days	*%*	*g*	*ppm*	*%*	*ppm*	*%*	*%*	*%*
Deficient	10	7.2 ± 0.7 **	111(36)	7.3 ± 0.3 **	38.1 ± 1.2	—	—	—	—
Control	10	12.4 ± 0.6	152(44)	28.2 ± 0.5	39.3 ± 1.1	—	—	—	—
Deficient	20	8.2 ± 0.4 **	151(20)	5.5 ± 0.7 **	37.6 ± 1.4	17.1 ± 2.1 **	34.9 ± 1.1	17.7 ± 0.7	0.4 ± 0.01 **
Control	20	13.6 ± 0.6	238(34)	15.5 ± 1.8	40.5 ± 0.6	36.7 ± 5.3	34.6 ± 0.7	17.5 ± 0.6	0.5 ± 0.02

[1] Milligram dried residue after alkali extraction per milligram aortic wet weight × 100. Mean ± SE of six observations.
[2] Number of chicks in parentheses.
[3] Expressed as micrograms copper per gram freeze-dried tissue.
[4] Percentage ash of fat-free dried bone.
[5] Mean ± SE of 10 observations.
[6] Mean ± SE of six observations.
** Significantly different from corresponding control ($P < 0.01$).

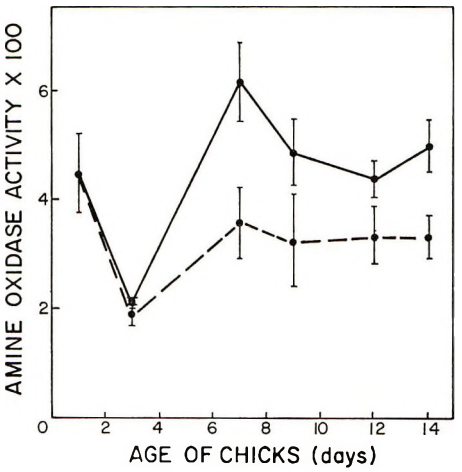

Fig. 1 Bone amine oxidase activity of copper deficient and control chicks. Each point and bar represents the average of five determinations ± SE. Values from controls are indicated as ●———●, and values from copper deficient chicks as ●– – – –●. Activity is expressed as μmoles benzaldehyde per hour per milligram protein × 100. The 10,000 × *g* supernates from bone homogenates were used in the assay.

sumed to be a dilution of activity due to the presence of such precursors.

It has been demonstrated that the ease of solubilization of collagen is related to the degree of intra- and intermolecular cross-linking (27). It has also been demonstrated that extraction of collagen in the presence of strong denaturants or neutral salts at elevated temperatures solubilizes these cross-linked polymers as gelatins (21). By all extraction methods used in these studies, the collagen from deficient chicks was more soluble than collagen from control chicks (fig. 3, table 2). The integrity of bone collagen appeared also to be closely associated to that of the mineral phase. The collagen from bone that was demineralized before or during collagen extraction was more readily solubilized than that from mineralized bone. It has been demonstrated by Stevens (28) and Glimcher et al. (29) that calcium protects collagen from solubilization. Food restriction has been shown to result in a less polymerized acid soluble collagen in rat skin (30). The possibility that some of the effects on collagen could have resulted from

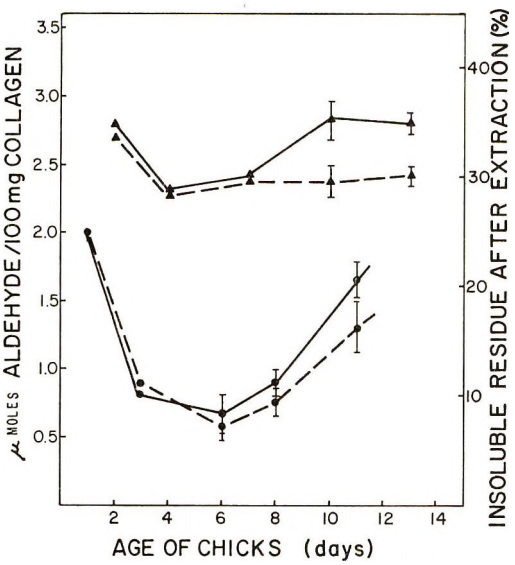

Fig. 2 Effect of copper deprivation on the aldehyde content of collagen and the percentage insoluble residue after guanidine and EDTA extraction of chick bone. The percentage insoluble residue is expressed as the milligram residue per milligram freeze-dried bone × 100. Values for aldehyde (μmoles/100 mg collagen) are indicated as ●———● for controls and ●- - - -● for copper-deficient chicks. Values for the percentage insoluble residue are indicated as ▲———▲ for controls and ▲- - - -▲ for copper-deficient chicks. Points and bars represent the mean of five determinations ± SE. Standard errors for insoluble residue at early ages were omitted for clarity because the points were close together.

the poorer growth of the copper-deficient chicks must be considered. This suggests the need for pair-fed or weight-paired controls in future work.

Cytochrome oxidase activity was reduced significantly in the mitochondrial fraction of bone from copper-deficient chicks when compared with controls (table 3). Bone catalase activity was not altered by copper intake. Catalase was determined, since copper deprivation affects the level of this enzyme in some tissues (31). At 20 days, amine oxidase activity of bone from copper-deficient chicks was again lower than that from control chicks. Furthermore, the dependence of bone amine oxidase activity on the bone copper level was demonstrated by the fact that addition of copper (0.5 ppm) to bone homogenates from copper-deficient birds completely restored activity

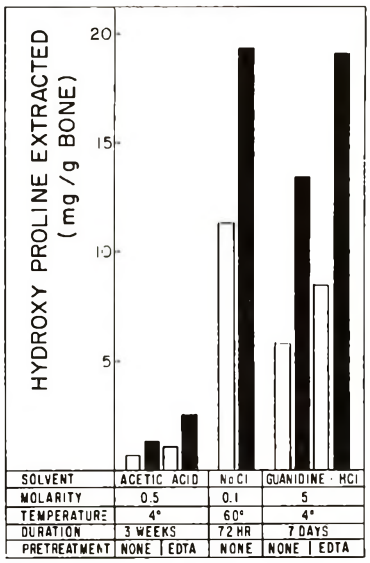

Fig. 3 Effects of demineralization and type of solvent on collagen extraction are demonstrated with bones from copper-deficient and control chicks. The material used for extraction in all cases was freeze-dried diaphyseal bone from 20-day-old copper-deficient and control chicks. The dark bars indicate values from copper-deficient bones and the light bars indicate values from control bones.

TABLE 2

Effect of copper-deficiency on alkali-soluble and insoluble collagen and aldehyde content of soluble collagen

Treatment [1]	Soluble collagen [2]	Insoluble collagen [2]	Aldehyde content [3]
	%	%	*μmoles/100 mg*
Control	0.27 ± 0.03(8) [4]	23.2 ± 1.3(8)	2.04 ± 0.18(3)
Deficient	0.44 ± 0.08(8) [5]	20.0 ± 0.5(8) *	1.47 ± 0.10(3) [5]

[1] Chicks were killed at 20 days on experimental treatments.
[2] Expressed as milligrams collagen per milligram freeze-dried bone × 100.
[3] Determination from acetic acid soluble collagen (19) on samples pooled from two or three chicks.
[4] Mean ± SE (number in parentheses represents number of determinations).
[5] Differed from corresponding control with a $P < 0.10$.
* Significantly different from corresponding control ($P < 0.05$).

TABLE 3

Effect of copper deficiency on bone enzymes

Treatment	Number of chicks [1]	Cytochrome oxidase [2]	Amine oxidase [3]	Catalase [4]
Control	10	0.70 **	1.62 **	9.8 ± 1.9
Deficient	10	0.38	1.00	10.8 ± 1.5
Deficient + copper [5]	5	—	1.65 **	—

[1] The chicks were 20 days of age and had been fed the experimental diets since 1 day old.
[2] Expressed as log [cytochrome c] per minute per milligram protein.
[3] Expressed as μmoles benzaldehyde per hour per milligram protein × 100.
[4] Expressed as Δ A/minute × 1000 ÷ 43.6 × milligrams protein per milliliter.
[5] Copper (0.5 ppm) added to the incubation mixture.
** Significantly different from copper deficient ($P < 0.01$).

to that of control chicks. Thus, it was demonstrated that some but not all of the effects of copper deficiency on enzyme activity previously shown for other tissues were also present in bone.

As indicated in this report, copper deprivation reduced the bone tissue level of copper. Furthermore, much of this copper could not be extracted with EDTA. EDTA treatment extracted over 96% of the bone mineral, but only 62 and 38% of the copper was extracted from control and copper-deficient bones, respectively. Hill (32) has also determined the copper content of chick bone, and likewise assumes copper to be associated with organic phase, because of the high stability constant (33) for the EDTA-copper complex (log K = 18). The copper remaining after EDTA extraction in deficient bone (10.9 ± 1.8 ppm) was not significantly different from that of control bone (14.2 ± 2.3) and probably represents copper which serves a structural rather than enzymatic role in the development of the bone organic matrix.

LITERATURE CITED

1. O'Dell, B. L., B. C. Hardwick, G. Reynolds and J. E. Savage 1961 Connective tissue defect in the chick resulting from copper deficiency. Proc. Soc. Exp. Biol. Med., *108:* 402.
2. Starcher, B., C. H. Hill and G. Matrone 1964 Importance of dietary copper in the formation of aortic elastin. J. Nutr., *82:* 318.
3. Carlton, W. W., and W. Henderson 1963 Cardiovascular lesions in experimental copper deficiency in chickens. J. Nutr., *81:* 200.
4. Savage, J. E., D. W. Bird, G. Reynolds and B. L. O'Dell 1966 Comparison of copper deficiency and lathyrism in turkey poults. J. Nutr., *88:* 15.
5. Carlton, W. W., and W. Henderson 1964 Skeletal lesions in experimental copper-deficiency in chickens. Avian Dis., *8: 48.*
6. Miller, E. J., G. R. Martin and K. A. Piez 1964 The utilization of lysine in the biosynthesis of elastin cross-links. Biochem. Biophys. Res. Commun., *17:* 248.
7. Harding, J. J. 1965 The unusual links and cross-links of collagen. Advance. Protein Chem., *20:* 109.
8. Bornstein, P., A. H. Kang and K. A. Piez 1966 The nature and location of intramolecular cross-links in collagen. Proc. Nat. Acad. Sci. US., *55:* 417.
9. Rojkind, M., L. Rhi and M. Aguirre 1967 Biosynthesis of the intermolecular cross-links in rat skin collagen. J. Biol. Chem., *243:* 2266.
10. Partridge, S. M., D. F. Elsdan, J. Thomas, A. Dorfman, A. Telser and P. L. Ho 1964 Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin. Biochem. J., *93:* 30C.
11. Chou, W. S., J. E. Savage and B. L. O'Dell 1968 Relation of monoamine oxidase activity and collagen cross-linking in copper-deficient and control tissues. Proc. Soc. Exp. Biol. Med., *128:* 948.
12. McGavack, T. H., and K. Y. T. Kao 1960 The influence of age and sex on the soluble collagen, insoluble collagen, and elastin of rat tissue. Exp. Med. Surg., *18:* 104.
13. Gorkin, V. Z., I. T. Grindeva, L. A. Romanova and I. S. Sererino 1962 Spectrophotometric determination of monoamine oxidase activity in mitochondria. Biokhimiya, *27:* 1004.
14. Blaschko, H. 1963 Amine oxidase. In: The Enzymes, vol. 8, eds., P. O. Boyer, H. Lardy and K. Myrback. Academic Press, New York, p. 337.
15. Copperstein, S. J., and A. Lazarow 1951 A microspectrophotometric method for the determination of cytochrome oxidase. J. Biol. Chem., *189:* 665.
16. Beers, R. F., Jr., and I. W. Sizer 1952 A spectrophotometric method for measuring the breakdown of H_2O_2 by catalase. J. Biol. Chem., *195:* 133.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randell 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
18. Kao, J-Y. T., W. E. Hitt, R. L. Dawson and T. H. McGavack 1962 Connective tissue. VII. Changes in protein and hexosamine content

of bone and cartilage of rats. Proc. Soc. Exp. Biol. Med., *110:* 538.
19. Fitch, S. M., M. Harkness and R. D. Harkness 1955 Extraction of collagen from tissues. Nature, *176:* 163.
20. Woessner, J. F. 1961 The determination of hydroxyproline in tissue and protein samples. Arch. Biochem. Biophys., *93:* 440.
21. Glimcher, M. J., and E. P. Katz 1965 The organization of collagen in bone: The role of noncovalent bonds in the relative insolubility of bone collagen. J. Ultrastruct. Res., *12:* 705.
22. Miller, E. J., G. R. Martin, K. A. Piez and M. J. Powers 1967 Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem., 242: 5481.
23. Paz, M. A., O. O. Blumenfeld, M. Rojkind, E. Hensor, C. Furfine and P. M. Gallop 1965 Determination of carbonyl compounds with *N*-methyl benzothiazolone hydrazone. Arch. Biochem. Biophys., *109:* 548.
24. Fiske, C. H., and Y. Subbarow 1925 Colorimetric determinations of phosphorus. J. Biol. Chem., 66: 375.
25. Allen, J. E. 1961 The determination of Cu by AAS. Spectrochim. Acta, *17:* 459.
26. Kim, C. S., and C. H. Hill 1966 The interrelationship of dietary copper and amine oxidase in the formation of elastin. Biochem. Biophys. Res. Commun., *24:* 395.
27. Piez, K. A. 1968 Cross-linking of collagen and elastin. Ann. Rev. Biochem., *37:* 547.
28. Stevens, F. S. 1967 The effect of chelating agents on collagen interfibrillar matrix interactions in connective tissue. Biochim. Biophys. Acta, *140:* 522.
29. Glimcher, M. J., U. A. Friberg, S. Orloff and J. Gross 1965 The role of the inorganic crystals in the solubility characteristics of collagen in lathyritic bone. J. Ultrastruct. Res., *15:* 74.
30. Deyl, Z., A. Everitt and J. Rosmus 1968 Changes in collagen cross-linking in rats with reduced food intake. Nature, *217:* 670.
31. Gubler, C. J., G. E. Cartwright and M. Wintrobe 1957 Enzyme activities and iron metabolism in copper and iron deficiencies. J. Biol. Chem., *224:* 533.
32. Hill, R. 1964 The manganese and copper of chick bone. Brit. J. Nutr., *19:* 163.
33. Stary, J. 1964 The Solvent Extraction of Metal Chelates. Macmillan Company, New York, p. 28.

Dietary Regulation of Transketolase Activity in Liver [1]

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ABSTRACT The effect of varying the dietary intake of thiamine, protein, fat and carbohydrate on the activity of transketolase in liver was studied in weanling rats. Maximum levels of transketolase activity were observed when the diet contained 0.75 mg or more of thiamine hydrochloride per 1000 kcal. It was found that when dietary thiamine is not limiting, the carbohydrate content of the diet appears to determine the level of activity of this enzyme. Of the carbohydrates tested, fructose induced the highest levels of transketolase activity. When either fat or protein was substituted for carbohydrate in the diet, liver transketolase activity decreased. This finding helps to explain why a high fat content in the diet has a thiamine "sparing" action. Like transketolase, hexose monophosphate (HMP) shunt dehydrogenase activity (combined glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities) increased in response to a high carbohydrate diet and decreased with a high fat diet. In contrast to transketolase, however, HMP shunt dehydrogenase activity increased in response to a high protein diet. This divergent response between transketolase and HMP shunt dehydrogenase suggests that the genes for the enzymes of the HMP shunt are not all located on the same operon.

Major alterations in the levels of activity of a variety of enzyme systems, with an attendant modification of metabolic patterns, can occur in the livers of animals subjected to changes in dietary composition (1–3). One such adaptation which has been studied in detail involves the two hexose monophosphate shunt (HMP) dehydrogenases — glucose 6-phosphate dehydrogenase (Glc-6-P DH) and 6-phosphogluconate dehydrogenase (6-PG DH). It has been shown that the levels of Glc-6-P DH and 6-PG DH are markedly influenced by the type and amount of carbohydrate in the diet (4–10). This finding has important implications relative to the capacity for generation of $NADPH_2$ and the formation of ribose (11, 12). Comparatively little information is available regarding the influence of dietary composition on other enzymes of the HMP shunt (13–15). It appeared of interest, in considering gene control of shunt activity, to determine whether the enzymes of the shunt are induced as a group or respond individually to different dietary stimuli. To obtain information regarding this point, the responses of transketolase activity in liver to variations in dietary protein, fat and carbohydrate were measured and compared with the concomitant changes in HMP shunt dehydrogenase activity. In the initial phase of this work, a specific and sensitive microfluorometric method for the measurement of transketolase activity was developed,[2] and the level of dietary thiamine needed for maximal transketolase activity was determined.

EXPERIMENTAL

Animals. Female rats of the Sprague-Dawley strain weighing 50 to 60 g were housed individually in wire-bottomed cages and were fed Purina Laboratory Chow [3] for 5 days during adaptation to the environment. Rats of comparable weights were then assigned randomly to the different dietary groups and were pair-fed for 2 weeks. Water was supplied ad libitum. Body weights and food consumed were recorded daily. All rats were fasted 24 hours before they were decapitated. Livers were quickly excised in a cold room (4°); they were rinsed with a 0.25 M sucrose solution,

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[2] Cheng, C. H., and R. E. Shank 1965 A microfluorometric method for the determination of transketolase activity in rat liver. Federation Proc., 24: 690 (abstract).
[3] Ralston Purina Company, St. Louis, Mo.

blotted with filter paper, weighed and stored at −70°. All assays were performed within 1 month of storage.

Diets. The basal diet contained 73% glucose, 18% vitamin-free casein, 4% corn oil, 4% Hegsted salt mixture (16), 1% cod liver oil, with the following added vitamins: (milligrams per kilogram) thiamine·HCl, 4; riboflavin, 8; pyridoxine·HCl, 4; Ca pantothenate, 25; niacin, 40; and choline chloride, 1000 (17). This diet was used throughout the experiments. Various modifications are described in the appropriate tables.

Tissue preparation and assay procedure. A 10% (w/v) homogenate in ice-cold water was made from 300 to 500 mg of frozen liver immediately prior to the enzyme assay. It was diluted to 0.2% (w/v) with 100 mM imidazole buffer, pH 7.6, containing 0.05% bovine plasma albumin.

Transketolase assays were performed in triplicate. Five microliters of 0.2% homogenate were added to 35 μl of an incubation mixture composed of imidazole buffer, 100 mM (pH 7.6); ribose-5-phosphate, 0.6 mM; sodium amytal, 1 mM; bovine plasma albumin, 0.05%; $NADH_2$, 0.1 mM; glycerophosphate dehydrogenase, 3×10^{-3} EU; triose phosphate isomerase, 4×10^{-2} EU; and pancreatic preparation of pentose isomerase and epimerase, 2×10^{-3} EU (18), in a 50 mm by 6 mm tube in an ice bath. The tube was incubated at 30° for 15 minutes. The reaction was stopped by returning the tubes to the ice bath and adding 4 μl of 5 N HCl. After mixing and standing at room temperature for 15 minutes, a 10-μl aliquot was mixed with 100 μl of 6.6 N NaOH in a 3-ml fluorometer tube and then incubated at 38° for 30 minutes. One milliliter of water was added and the fluorescence of alkaline derivative of NAD was read after the tube was cooled to room temperature. The primary filter of the fluorometer was Corning no. 5860 [4] and secondary filters were nos. 5562, 4308 and 3387 as recommended by Lowry et al. (19). Tissue blanks and internal standards were used to correct for any nonspecific activity. The activities are given as millimoles of NAD formed per kilogram wet weight of liver per hour.

The method for the combined determination of Glc-6-P and 6-PG dehydrogenases was a modification of procedures in the literature (20–22). NADP reduction was measured at 340 mμ in a spectrophotometer [5] in cuvettes of 1-cm light path, at 38°. One milliliter of reaction mixture contained: bovine plasma albumin, 0.02%; $MgCl_2$, 10 mM; glucose 6-phosphate (disodium salt), 3.0 mM; 6-phosphogluconate (tri-sodium salt), 3.0 mM; NADP (sodium salt), 0.6 mM, in 100 mM imidazole (Sigma, grade III [6]) buffer, pH 7.6; and homogenate of 0.1 to 0.25 mg wet weight of liver. Readings were taken at 1-minute intervals starting immediately after the addition of glucose 6-phosphate and 6-phosphogluconate. The combined activities of the two dehydrogenases are given as moles of $NADPH_2$ formed per kilogram of wet weight of liver per hour.

Protein content of the liver was determined by the method of Lowry et al. (23).

All data were treated with analysis of variance followed by Duncan's new multiple range test (24), and results are expressed as mean values ± the standard error.

RESULTS AND DISCUSSION

Thiamine pyrophosphate is the coenzyme of transketolase (25, 26). It had been shown that varying the dietary content of thiamine influences the activity of this enzyme in tissue (15, 17, 27, 28). Studies were therefore conducted to determine the thiamine level of the diet needed to ensure that the vitamin would not be a limiting factor when other dietary ingredients were varied.

As shown in table 1 (exp. 1), increasing the thiamine content above 4 mg/kg of diet had no effect on rat liver transketolase activity. A significantly lower level of enenzyme activity, however, was found when thiamine content was reduced to 2 mg/kg of diet. To determine the minimum dietary thiamine level which would provide maximum transketolase activity, the experiment was repeated with thiamine contents in the diets varying between 1 mg/kg and 4 mg/kg. As shown in experiment 2 of table 1, transketolase activity was the same in the liver of rats fed 3 or 4 mg thiamine·

[4] Corning Glass Works, Inc., Corning, N. Y.
[5] Beckman model DU, Beckman Instruments, Inc., Fullerton, Calif.
[6] Sigma Chemical Company, St. Louis, Mo.

TABLE 1

Effect of various levels of dietary thiamine on hepatic transketolase activity

Exp. no.	Diet group [1]	Added thiamine in diet	No. of rats	Body wt before killing	Liver wt	Liver protein	Transketolase activity [2]
		mg/kg		*g*	*g*	*%*	*mmoles/kg per hr*
1	A	2	6	122.5	4.29	21.1 ± 0.7	170.7 ± 5.4
	B	4	6	116.2	3.93	21.0 ± 0.6	216.3 ± 4.4
	C	20	6	117.8	3.85	21.7 ± 0.3	214.6 ± 6.4
	D	50	6	112.0	3.53	22.1 ± 0.3	213.9 ± 7.2
2	1	1	6	146.3	4.84	20.7 ± 0.2	38.8 ± 13.2
	2	2	5 [3]	154.6	4.19	20.5 ± 0.3	150.7 ± 19.4
	3	3	6	168.5	4.58	21.6 ± 0.5	194.1 ± 18.6
	4	4	6	163.7	4.52	20.9 ± 0.7	199.9 ± 7.6

[1] Groups B, C and D were pair-fed with group A in experiment 1. Average food intake was 12 g/rat per day. Groups 2, 3 and 4 were pair-fed with group 1 in experiment 2. Average food intake in this experiment was 13 g/rat per day.

[2] Transketolase activity:
A versus B or C or D: $P < 0.01$. 1 versus 2 or 3 or 4: $P < 0.01$. 2 versus 3 or 4: $P < 0.05$.

[3] One rat died of unknown cause.

HCl/kg diet, but was significantly lower in liver of rats afforded 1 and 2 mg thiamine·HCl/kg diet. Byerrum and Flokstra (29) used a diet similar to ours and determined the thiamine and cocarboxylase content of liver, muscle and brain of rats. They found that as the level of thiamine was increased to 0.2 mg/100 g diet, maximum values for cocarboxylase content in tissue were achieved. The highest thiamine level in tissues was found when dietary thiamine was increased to 0.4 mg/100 g. Our data indicated that maximum liver transketolase activity was reached when the dietary thiamine level was 3 mg/kg or 0.3 mg/100 g. Since the total caloric value of the basal diet is 4000 kcal/kg, the minimum dietary thiamine level in this diet, to provide maximum liver transketolase activity, would be close to 0.75 mg/1000 kcal. Therefore, to insure that dietary thiamine was adequate, thiamine·HCl was added to the diet, used in subsequent studies at the level of 4 mg/kg or 1.0 mg/1000 kcal.

The effect of the quality of dietary protein on liver transketolase activity was studied by comparing diets containing either methionine-supplemented casein or gelatin. The results in table 2 clearly indicate the superiority of the casein diet. This difference is probably due to the fact that gelatin is inadequate in tyrosine and cystine and is completely lacking in tryptophan (30) which is essential for tissue protein synthesis. The loss of body weight, the low liver weight, the decreased total liver protein, are all compatible with the effects expected from an incomplete protein.

The quantitative effect of dietary casein on transketolase in liver is shown in table 3. In this experiment the casein contents of the diets were modified from the basal diet by isocaloric interchange with the glucose in the respective diet. The significantly lower transketolase activity in the livers of rats on the protein-deficient diet is probably due to the lack of essential amino acids. Interestingly, a two- to fourfold increase of dietary protein above the control level, with a concomitant reduction in carbohydrate, caused a marked decrease in transketolase activity. Moreover, no significant change in liver protein content was found among the three groups.

The effect of varying the type of fat in the diet on liver transketolase activity is shown in table 4. Cottonseed oil, butter, coconut oil and lard were substituted for the 4% corn oil of the basal diet. These fats were chosen not only because they are commonly used, but also because they have different fatty acid compositions. Nevertheless, they caused no significant differences in hepatic transketolase activity over a period of 2 weeks.

It has been emphasized that fat has a sparing action on thiamine (31–33). Balaghi and Pearson (31) reported that a significantly higher percentage of the thiamine eaten by rats is excreted in the urine

TABLE 2

Effect of the quality of dietary protein on liver transketolase activity

Diet group [1]	Dietary protein	No. of rats	Body wt gain	Liver wt	Total liver protein	Transketolase activity [2]
			g	*g*	*mg*	*mmoles/kg per hr*
C	Casein [3]	6	8.0	3.14	687.3	181.9 ± 3.2
G	Gelatin [4]	6	−32.9	2.21	491.9	109.1 ± 3.8

[1] Group C was pair-fed with group G. Average caloric intake was 32 kcal/rat per day.
[2] C versus G: $P < 0.01$.
[3] Vitamin-free casein used in this diet was supplemented with methionine at a level of 5%.
[4] Eighteen percent gelatin as sole source of dietary protein.

TABLE 3

Effect of different proportions of protein in the diet on liver HMP shunt enzyme activity

Diet group [1]	Dietary protein content [2]	No. of rats	Body wt gain	Liver wt	Liver protein content [3]	Transketolase activity [4]	Glc-6-P DH and 6-PG DH activity [5]
	%		*g*	*g*	%	*mmoles/kg per hr*	*moles/kg per hr*
D	0	6	−21.1	1.47	18.2 ± 0.8	116.1 ± 8.9	0.389 ± 0.022
C	18	6	17.7	2.99	20.7 ± 0.6	171.3 ± 13.2	0.860 ± 0.042
2X	36	6	22.8	2.92	21.6 ± 0.4	90.8 ± 6.2	0.973 ± 0.037
4X	72	6	22.2	3.47	21.3 ± 0.6	71.3 ± 6.9	1.323 ± 0.091

[1] Groups C, 2X and 4X were pair-fed with group D. Average food intake for each group was 8 g/rat per day.
[2] Protein was isocalorically interchanged with glucose in the basal diet.
[3] Protein content:
D versus 2X, 4X: $P < 0.01$. D versus C: $P < 0.05$.
[4] Transketolase activity:
C versus D, 2X, 4X: $P < 0.01$. D versus 4X: $P < 0.01$. D versus 2X: $P < 0.05$.
[5] Dehydrogenases activity: Differences among all means are highly significant ($P < 0.01$) except that between C and 2X.

TABLE 4

Effect of different fats and oils in the diet on liver transketolase activity

Diet group [1]	Dietary fat or oil	No. of rats	Body wt gain	Liver wt	Liver protein content	Transketolase activity [2]
			g	*g*	%	*mmoles/kg per hr*
CR	Corn oil	6	35.3	3.44	20.1 ± 0.3	152.0 ± 8.6
CS	Cottonseed oil	6	42.2	3.92	20.2 ± 0.3	166.3 ± 11.9
B	Butter	6	41.5	3.76	19.9 ± 0.4	161.6 ± 5.3
CC	Coconut oil	6	38.9	3.76	19.8 ± 0.4	154.3 ± 10.6
L	Lard	5 [3]	37.4	3.54	20.0 ± 0.5	151.6 ± 14.2

[1] Groups CR, CS, CC and L were pair-fed with group B. Average food intake was 12 g/rat per day.
[2] No significant differences were found among the groups.
[3] One rat in this group died of pneumonia.

when they are on a high fat diet compared with a high carbohydrate diet. These findings motivated us to determine whether varying the amount of fat in the diet would also affect transketolase which has thiamine pyrophosphate as coenzyme. Our data in table 5 indicate that there was no significant difference between levels of hepatic transketolase activity of rats given a control diet containing 5% corn oil, or of rats on a diet entirely free of fat for 2 weeks, as long as the carbohydrate content is high. When the fat content of the diet was increased to 11%, however, with an isocaloric reduction in glucose, a highly significant decrease of transketolase activity occurred ($P < 0.01$). Further increases in fat and decreases in carbohydrate content resulted in an even greater reduction in transketolase activity (table 5). These findings suggested the possibility that the decrease in carbohydrate in the diet resulted in a decrease in the inducer for transketolase. The reduction in the level

of transketolase resulted in a decrease in the number of specific binding sites for the coenzyme thiamine pyrophosphate. Therefore, with fewer such sites available, less thiamine should be needed to saturate the binding sites on transketolase. If other enzymes containing thiamine pyrophosphate as cofactor also react similarly, this might help to explain the mechanism by which thiamine requirement is closely related to carbohydrate in the diet, and why fat has a sparing action on thiamine. Baldwin and co-workers (5) reported a decrease in the activity of Glc-6-P DH, 6-PG DH and pentose-phosphate-metabolizing enzyme when lard was substituted for glucose. Freedland and Barnes (6) found that when margarine replaced dextrose in the diet, Glc-6-P DH activity was reduced. Our finding that the isocaloric replacement of dietary carbohydrate with either protein or fat caused a significant decrease in liver transketolase activity suggests that when dietary thiamine is adequate, the level of this enzyme is controlled by the amount of carbohydrate in the diet.

To obtain information regarding the effects of different types of carbohydrates on transketolase activity, the responses to diets in which glucose was completely replaced with either fructose, sucrose or potato starch were studied. As shown in table 6, the diet containing potato starch resulted in a significant reduction in transketolase activity, whereas sucrose and fructose resulted in a significant increase. The latter observation provided evidence that fructose was a more effective inducer of transketolase activity than glucose. This was of particular interest in view of the marked effect of dietary fructose on the HMP shunt dehydrogenases (4, 7–9).

The observation that the level of liver transketolase activity in rats fed potato starch was significantly lower than that found in rats fed glucose was somewhat

TABLE 5

Effect of different proportions of fat in the diet on liver HMP shunt enzyme activity

Diet group [1]	Fat content in diet [2]	No. of rats	Body wt gain	Liver wt	Liver protein content	Transketolase activity [3]	Glc-6-P DH and 6-PG DH activity [4]
	%		*g*	*g*	%	*mmoles/kg per hr*	*moles/kg per hr*
A	0	6	50.8	4.31	20.04 ± 0.59	182.6 ± 10.7	4.310 ± 0.250
B	5	6	49.0	4.10	20.40 ± 0.62	195.9 ± 7.1	2.290 ± 0.170
C	11	6	50.9	4.37	20.36 ± 0.52	162.4 ± 5.9	1.600 ± 0.110
D	25	6	46.9	4.16	20.49 ± 0.63	156.1 ± 3.0	0.990 ± 0.080
E	62	6	67.3	5.13	19.80 ± 0.41	85.5 ± 4.5	0.740 ± 0.050

[1] Groups A, B, C and D were pair-fed with group E. Average caloric intake was 46 kcal/rat per day.

[2] Diet B in this experiment contained per kilogram: 20,000 IU vitamin A; 2,500 IU vitamin D; 150 mg α-tocopherol; 1000 mg choline chloride; 4 mg thiamine·HCl; 8 mg riboflavin; 4 mg pyridoxine; 25 mg Ca pantothenate; 40 mg niacin; 40 g salt mixture (Hegsted); 180 g protein; and 720 g glucose. The other diets were prepared by isocalorically substituting fat for glucose. To maintain the consistency of the diet, half of the above weights of the fat was afforded as hydrogenated corn oil and half as liquid corn oil.

[3] Transketolase activity:
B versus C, D, E: $P < 0.01$. A versus D: $P < 0.05$. E versus A, B, C, D: $P < 0.01$.

[4] Dehydrogenases activity: All differences between means are significant: $P < 0.01$.

TABLE 6

Effect of different dietary carbohydrates on liver HMP shunt enzyme activity

Diet group [1]	Dietary carbohydrate	No. of rats	Body wt gain	Liver wt	Liver protein content [2]	Transketolase activity [3]	Glc-6-P DH and 6-PG DH activity [4]
			g	*g*	%	*mmoles/kg per hr*	*moles/kg per hr*
F	Fructose	6	15.0	3.30	21.1 ± 0.4	254.9 ± 14.3	1.830 ± 0.065
S	Sucrose	6	20.2	3.20	20.4 ± 0.3	233.3 ± 15.3	1.732 ± 0.085
G	Glucose	6	16.0	2.83	21.1 ± 0.4	179.8 ± 7.1	1.217 ± 0.099
P	Potato starch	5 [5]	−6.6	1.87	20.0 ± 0.9	110.8 ± 9.3	0.620 ± 0.062

[1] Groups F, S, G were pair-fed with group P. Average food intake was 8 g/rat per day.

[2] There were no significant differences between the means of protein content in liver.

[3] All differences between means are significant ($P < 0.01$) except that between F and S which is not significant.

[4] All differences between means are significant ($P < 0.01$) except that between F and S.

[5] One rat died due to severe diarrhea and weight loss.

unexpected, since starch is broken to glucose before absorption. A possible explanation is that animals fed glucose, which is rapidly absorbed, developed higher concentrations of glucose in the portal vein blood and therefore in the hepatic cells following a meal than do animals fed potato starch, from which glucose is released and absorbed more gradually and incompletely.

The observation that transketolase, like Glc-6-P DH and 6-PG DH, appears to be induced by fructose raised the possibility that the genes for these HMP shunt enzymes might be located on the same operon and therefore respond in parallel to the same inducers and repressors. To test this possibility, the levels of HMP shunt dehydrogenase activity in the livers of the rats subjected to the above described dietary manipulations were measured.

The observation that fructose is a more effective inducer of Glc-6-P DH and 6-PG DH was confirmed (table 6). The HMP shunt dehydrogenase activity also decreased progressively as increasing amounts of dietary carbohydrate were replaced with fat (table 5). This is in keeping with the observations of Johnson and Sassoon (4), Fitch and Chaikoff (8), Tepperman and Tepperman (34) and others (5, 6, 35) that increasing the fat content of the diet decreased HMP dehydrogenase activity.

In contrast to the observed decrease of transketolase activity, substitution of increasing amounts of casein for glucose in the diet resulted in a concomitant increase in HMP shunt dehydrogenase activity (table 3). This observation agrees with the findings of Tepperman and Tepperman (10), Johnson and Sassoon (4), Freedland and Barnes (6), and provides strong evidence that transketolase and the HMP shunt dehydrogenases are not located on the same operon.

ACKNOWLEDGMENT

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LITERATURE CITED

1. Takeda, Y., H. Inoue, K. Honjo, H. Tanioka and Y. Daikuhara 1967 Dietary response of various key enzymes related to glucose metabolism in normal and diabetic liver. Biochim. Biophys. Acta, *136:* 214.
2. Ashida, K. 1963 Diets and tissue enzymes. In: Newer Methods of Nutritional Biochemistry, vol. 1, ed., Anthony A. Albanese. Academic Press, New York, p. 159.
3. Knox, W. E., V. H. Auebrach and C. C. Lin 1956 Enzymatic and metabolic adaptations in animals. Physiol. Rev., *36:* 164.
4. Johnson, B. C., and H. F. Sassoon 1967 Studies on the induction of liver glucose-6-phosphate dehydrogenase in the rat. In: Advances in Enzyme Regulation, vol. 5, ed., George Weber. Pergamon Press, New York, p. 93.
5. Baldwin, R. L., M. Ronning, C. Radanovics and G. Plange 1966 Effect of carbohydrate and fat intakes upon the activities of several liver enzymes in rats, guinea piglets, piglets and calves. J. Nutr., *90:* 47.
6. Freedland, R. A., and J. K. Barnes 1963 The effect of adrenalectomy on the adaptation of G-6-P metabolizing enzymes in the liver. J. Biol. Chem., *238:* 1915.
7. Weber, G., J. Ashmore and G. Banerjee 1960 Hormonal and dietary effects on hepatic carbohydrate enzymes. Federation Proc., *19:* 50.
8. Fitch, W. M., and I. L. Chaikoff 1960 Extent and patterns of adaptation of enzyme activities in livers of normal rats fed diets high in glucose and fructose. J. Biol. Chem., *235:* 554.
9. Fitch, W. M., R. Hill and I. L. Chaikoff 1959 The effect of fructose feeding on glycolytic enzyme activity of the normal rat liver. J. Biol. Chem., *234:* 1048.
10. Tepperman, J., and H. M. Tepperman 1958 Effect of antecedent food intake pattern on hepatic lipogenesis. Amer. J. Physiol., *193:* 55.
11. Racker, E. 1956 Micro- and macrocycles in carbohydrate metabolism. Harvey Lect., *51:* 143.
12. Racker, E. 1961 Transketolase. In: The Enzymes, vol. 5, eds., P. D. Boyer, H. Lardy and K. Myrbäck. Academic Press, New York, p. 397.
13. Benevenga, N. J., W. J. Stielau and R. A. Freedland 1964 Factors affecting the activity of pentose phosphate-metabolizing enzymes in rat liver. J. Nutr., *84:* 345.
14. Brin, M. 1963 Thiamine deficiency and erythrocyte metabolism. Amer. J. Clin. Nutr., *12:* 107.
15. Stielau, W. J., R. A. Freedland and J. H. Meyer 1965 Effects of B-vitamin deficiencies and of starvation on liver enzyme activities of growing rats. J. Nutr., *87:* 109.
16. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.
17. Brin, M. 1962 Effect of thiamine deficiency and of oxythiamine on rat tissue transketolase. J. Nutr., *78:* 179.
18. Ashwell, G., and J. Hickman 1957 Enzymatic formation of xylulose-5-phosphate from

ribose-5-phosphate in spleen. J. Biol. Chem., *226:* 65.
19. Lowry, O. H., N. R. Roberts and J. I. Kapphahn 1957 The fluorometric measurement of pyridine nucleotides. J. Biol. Chem., *224:* 1047.
20. Kornberg, A., and B. L. Horecker 1955 Glucose-6-phosphate dehydrogenase. In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 323.
21. Horecker, B. L., and P. Z. Smyrniotis 1955 6-Phosphogluconic dehydrogenase. In: Methods in Enzymology, vol. 1, eds., S. P. Colowick and N. O. Kaplan. Academic Press, New York, p. 323.
22. Marks, P. A. 1966 6-Phospho-gluconate dehydrogenase. In: Methods in Enzymology, vol. 9. Carbohydrate Metabolism, ed., Willis A. Wood. Academic Press, New York, p. 141.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with Folin phenol reagent. J. Biol. Chem., *193:* 265.
24. Steel, R., and J. Torrie 1960 Principles and Procedures of Statistics. McGraw-Hill Book Company, New York, p. 107.
25. Horecker, B. L., and P. Z. Smyrniotis 1953 The coenzyme function of thiamine pyrophosphate in pentose phosphate metabolism. J. Amer. Chem. Soc., *75:* 1009.
26. Racker, E., G. De LaHabe and I. G. Leder 1953 Thiamine pyrophosphate — a coenzyme of transketolase. J. Amer. Chem. Soc., *75:* 1010.
27. Brin, M. 1964 Erythrocyte as a biopsy tissue for functional evaluation of thiamine adequacy. J. Amer. Med. Assoc., *187:* 762.
28. Dreyfus, P. M. 1962 Clinical application of blood transketolase determinations. New Engl. J. Med., *267:* 596.
29. Byerrum, R. U., and J. H. Flokstra 1951 Cocarboxylase and thiamine in tissues of rats receiving different concentrations of vitamin B_1 in the diet. J. Nutr., *43:* 17.
30. Orr, M. L., and B. K. Watt 1957 Home Econ. Res. Rep. no. 4. U. S. Dep. Agr., Washington, D. C.
31. Balaghi, M., and W. N. Pearson 1966 Alterations in the metabolism of 2-^{14}C-thiazole-labeled thiamine by the rat induced by a high fat diet or thyroxine. J. Nutr., *90:* 161.
32. Evans, H. M., and S. Lepkovsky 1929 Sparing action of fat on the antineuritic vitamin B. J. Biol. Chem., *83:* 269.
33. DeCaro, L., and G. Rindi 1951 Sparing action of fats and pyruvate in the blood of rats in avitaminosis B_1. Nature, *167:* 114.
34. Tepperman, H. M., and J. Tepperman 1963 On the response of hepatic G-6-P DH activity to changes in diet composition and food intake pattern. Advances in Enzyme Regulation, vol. 1, ed., George Weber. Pergamon Press, New York, p. 121.
35. Reed, P. W., and J. Tepperman 1968 Hexosemonophosphate shunt dehydrogenase activity in rat liver and leukocyte: effect of diet and thyroid status. Proc. Soc. Exp. Biol. Med., *128:* 888.

Hepatic Tyrosine Transaminase Rhythm: Interaction of environmental lighting, food consumption and dietary protein content [1]

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ABSTRACT Hepatic tyrosine transaminase activity was measured over a 24-hour period in animals maintained under three environmental situations: normal lighting (lights on from 9 AM to 9 PM) and ad libitum dietary protein; reversed lighting (lights on from 9 PM to 9 AM) and ad libitum dietary protein; and normal lighting and a nonprotein diet, with protein added at various times during the day. In each case, the increase in tyrosine transaminase activity was not observed until after the initial ingestion of protein. These results support the hypothesis that the daily rhythm in the activity of this enzyme in rat liver is generated by the cyclical ingestion of protein.

The activity of the hepatic enzyme tyrosine transaminase varies fourfold as a function of the time of day (1–3). When rats are kept under normal environmental lighting (e.g., lights on from 6 AM to 6 PM) and given access to food and water ad libitum, enzyme activity is at a minimum early in the morning, begins to rise late in the afternoon and reaches a maximum several hours after the onset of darkness.

A similar rhythm exists in food intake (4). Rats eat relatively little until late in the light period, and consume most of their food in the hours following the onset of darkness. Rats acutely deprived of food show no rhythm in tyrosine transaminase activity (5). Moreover, rats fed a nonprotein diet also show no enzyme rhythm, although the feeding rhythm persists (5). It appeared, therefore, that the tyrosine transaminase rhythm is generated by the cyclical ingestion of dietary protein. To examine this hypothesis further, we altered the rhythm in protein intake and observed the consequent effects on the enzyme rhythm.

In the rat, the feeding rhythm is synchronized by light. An alteration in the time of the daily light exposure is followed within a few days by a similar change in the pattern of food intake. If an increase in the rate of protein consumption provides the necessary signal for the daily rise in tyrosine transaminase, such a change in the feeding rhythm should be accompanied by a parallel change in the enzyme rhythm. Similarly, if protein consumption is altered independently of the feeding rhythm (by giving rats maintained under a standard lighting schedule protein-free food ad libitum and then replacing this food with a protein diet at different times during the day), the rise in tyrosine transaminase activity should follow protein ingestion and not simply the feeding rhythm. Experiments are described which test these hypotheses.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain, weighing 180 to 200 g at the onset of the experiment, were obtained commercially.[2] Animals were housed individually and exposed to 50 to 70 ft-c of illumination from cool-white fluorescent bulbs. Measurements of food and water consumption were made by weighing the containers and correcting for the loss due to evaporation. At intervals during a 24-hour period,

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[2] Charles River Laboratories, Inc., Wilmington, Mass.

groups of three or four animals subjected to each experimental treatment were decapitated; the livers were removed and stored on dry ice until assayed. Tyrosine transaminase was assayed by a modification (1) of the method of Diamondstone (6). Diets containing zero percent or 18% protein were prepared as previously described (5).

RESULTS

In experiment 1, Purina Laboratory Chow [3] and water were available ad libitum, and food and water intake were measured at the beginning and end of the daily light period (9 AM to 9 PM). Food consumption showed a marked 24-hour rhythm; about 80% of the total daily intake was consumed during the dark period. The lighting schedule was then reversed; lights were now on from 9 PM to 9 AM. Animals were killed at intervals during day 3 or 6 of exposure to the new lighting regimen.

The rhythms in feeding behavior and tyrosine transaminase activity appeared to shift phase at the same rate: after 3 days under reversed lighting conditions, the rats ate 40% of their food during the dark period, and showed a 4- to 6-hour shift in the time of peak tyrosine transaminase activity. After 5 days of reversed lighting, rats ate 77% of their daily food intake during the new dark period, and on day 6 the peak in enzyme activity had shifted by a full 12 hours (fig. 1). Other animals killed after 11 days of exposure to reversed lighting showed a rhythm similar to that seen after 6 days, but with less variance.

In experiment 2, animals under a normal lighting schedule (lights on from 7 AM to 7 PM) previously given access to Purina Laboratory Chow were given a nonprotein diet beginning at 10 AM. This was replaced by an isocaloric diet containing 18% casein at 1 PM, 6 PM, 1 AM or not at all. The expected rhythm in food intake was observed in each group, although animals eating the protein-free diet ate somewhat less than animals given the diet containing protein (table 1).

The livers of rats which ate the nonprotein diet showed a low level of tyrosine transaminase activity throughout the 24-hour period; no marked rise in tyrosine transaminase occurred until after this diet was replaced by 18% casein. Thus, animals receiving protein at 1 PM showed a significant increase in enzyme activity by 6 PM, animals given protein at 6 PM had elevated tyrosine transaminase activity by 10 PM, and animals receiving protein at 1 AM showed a similar increase by 5 AM (fig. 2).

DISCUSSION

It has previously been suggested (5) that the daily rhythm in tyrosine transaminase activity resulted from the cyclical intake of dietary protein, and was not a direct response to the time of day, the light cycle, or an endogenous "biological clock." The experiments reported here support this hypothesis.

If the rhythm in food intake generates the rhythm in transaminase activity, experimental alterations of the former should be accompanied by simultaneous changes in the latter. This was observed in experiment 1: reversal of the light cycle was followed by coupled changes in the timing of the food and enzyme rhythms. Moreover, if the generating signal within food is protein, dissociating the time of day that the animal consumes protein and the time that it elects to eat should produce a parallel dissociation between the transaminase rhythm and the rhythm in food intake. In experiment 2, animals initially fed a nonprotein diet showed a feeding rhythm but no enzyme rhythm. The rise in enzyme activity was not observed until after protein was added to the diet.

Under normal laboratory conditions, the liver of the rat receives a large influx of dietary protein via the portal circulation for part of every 24-hour period. A parallel rhythm in tyrosine transaminase activity appears to be one of the adaptive responses of the liver to this influx (5, 7). Tyrosine transaminase activity is normally high during the time of day that the animal ingests tyrosine (i.e., in dietary protein) at the greatest rate. The enzyme rhythm may thus have the effect of dampening the large fluctuations which might otherwise occur in the tyrosine concentrations of blood or liver.

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

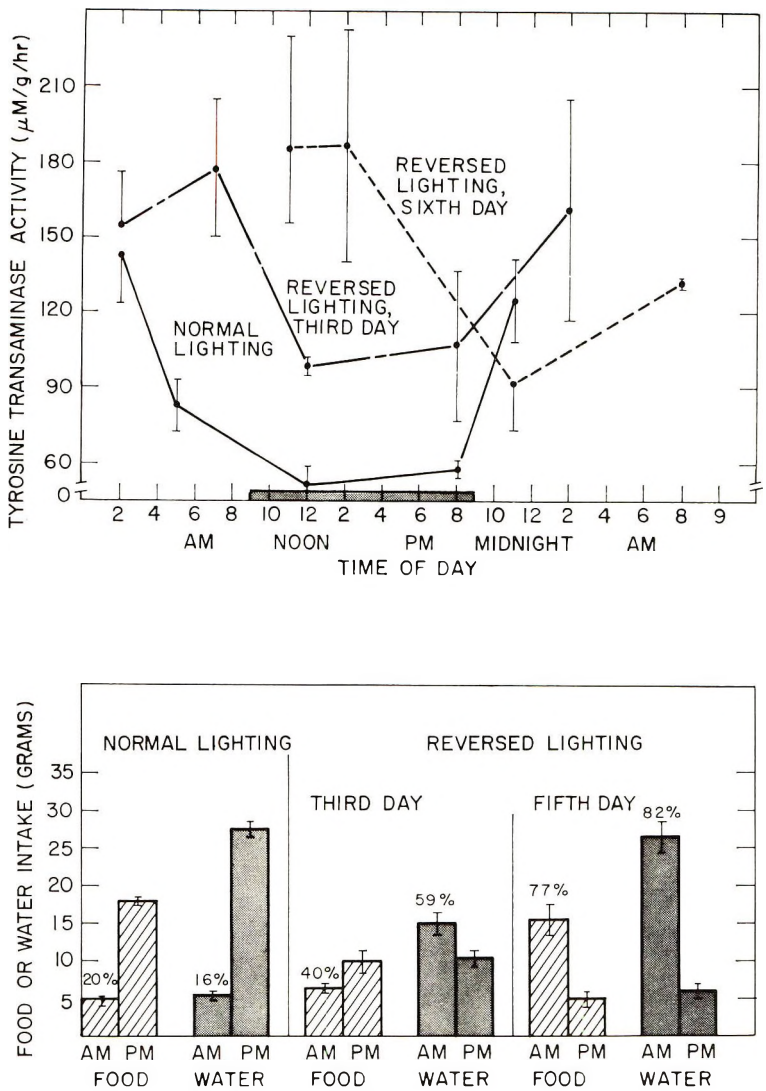

Fig. 1 Tyrosine transaminase activity and food and water intake in animals under normal (9 AM to 9 PM) or reversed (9 PM to 9 AM) lighting conditions. a) Tyrosine transaminase activity after 3 or 6 days of reversed lighting is compared with enzyme activity in rats kept under normal lighting. Each point represents the average of three animals. Shaded horizontal bar along abscissa indicates hours of darkness under new (i.e., reversed) lighting conditions. b) Food and water intake during the light and dark hours are compared for the last day of normal lighting, and days 3 and 5 of reversed lighting. Each bar represents the average of 12 (reversed lighting) or 30 (normal lighting) animals. Vertical lines represent the estimated standard error of the mean.

The tyrosine transaminase rhythm is a reflection of the laboratory feeding habits of the rat. An animal with a different feeding pattern (such as the human) might be expected to have a different enzyme rhythm. Although maximal hepatic enzyme activity is normally observed at night in the adult rat, it has been shown that rats fed during the hours of 8 AM and 12 noon have an early morning peak in enzyme activity (7) and that before weaning, the suckling rat (whose feeding activity is maximal during the early morning hours) shows maximal tyrosine transaminase ac-

TABLE 1

Rate of food intake in rats fed protein and nonprotein diets [1]

	10 AM to 1 PM	1 PM to 6 PM	6 PM to 10 PM	10 PM to 1 AM	1 AM to 5 AM	5 AM to 10 AM
	g/hr	*g/hr*	*g/hr*	*g/hr*	*g/hr*	*g/hr*
Nonprotein diet [2]	0.61 ± 0.21 [3]	0.53 ± 0.07	1.05 ± 0.17	1.02 ± 0.23	0.69 ± 0.13 [4]	
Protein from 1 PM	—	0.66 ± 0.11	1.10 ± 0.13	1.67 ± 0.22	0.92 ± 0.72	0.97 ± 0.15
Protein from 6 PM	—	—	1.22 ± 0.18	1.43 ± 0.26	1.92 ± 0.42	0.57 ± 0.23
Protein from 1 AM	—	—	—	—	2.38 ± 0.19	0.50 ± 0.40

[1] All animals received a nonprotein diet at 10 AM. This was replaced by a similar diet supplemented with 18% casein at 1 PM, 6 PM, 1 AM or not at all.
[2] Data represent average food intake of all animals receiving nonprotein diet during each time interval.
[3] Mean ± estimated SE of the mean.
[4] Food intake was measured only once between 1 AM and 10 AM in this group.

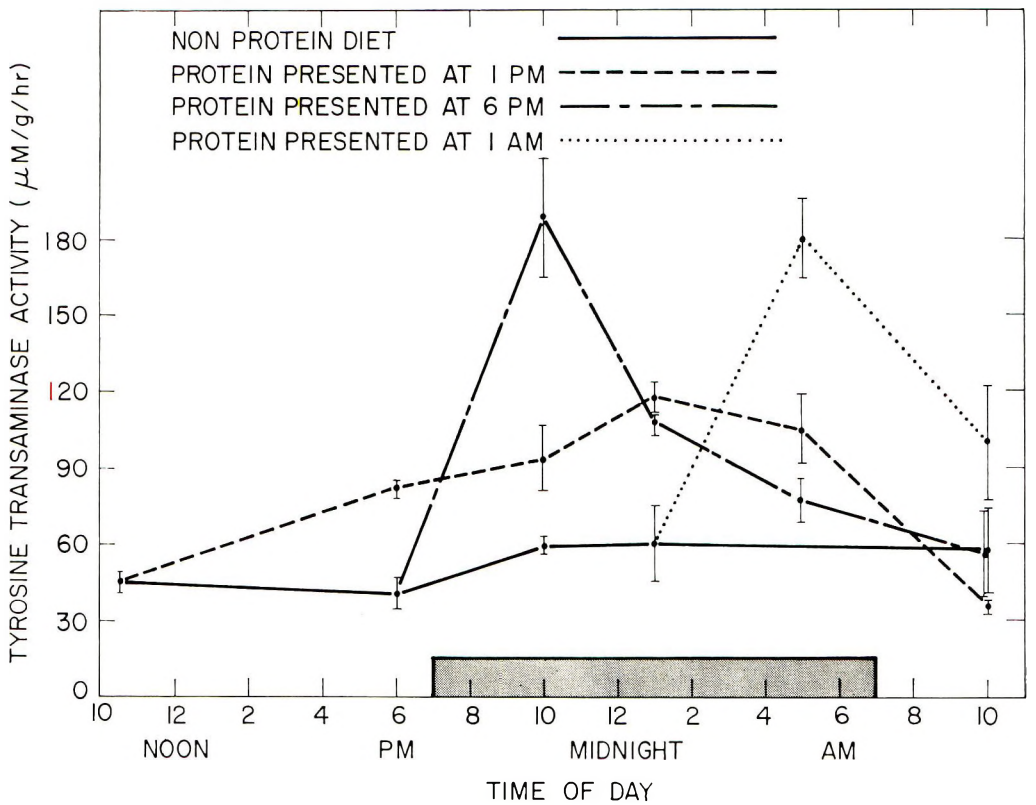

Fig. 2 Tyrosine transaminase activity in animals given a nonprotein diet at 10 AM, and then an 18% protein diet at 1 PM, 6 PM, 1 AM or not at all. Lights were on from 7 PM to 7 AM. Each point represents the average of three or four animals. Lines connect enzyme activity determined the first time after protein was made available with activity observed the previous time that it was sampled. Vertical lines represent the estimated standard error of the mean.

tivity during the light period (8). Preliminary data suggested that rats trained to eat at a constant rate throughout a 24-hour period would show no rhythm in tyrosine transaminase activity.[4]

Tyrosine transaminase activity increased only slowly among rats receiving protein at 1 PM. In contrast, rats first given pro-

[4] Cohn C., and R. J. Wurtman, unpublished observations.

tein 5 hours later showed a much faster rise (fig. 2). This difference may be related to the relative rates at which protein was initially ingested: the rate of food consumption is much slower in the afternoon than in the early evening (table 1). It might also result from an underlying rhythm in the extent to which the enzyme can be induced.

The small but significant variation in transaminase activity seen among animals receiving no protein (fig. 2) has been noted in previous experiments (4). Because the concentration of corticosterone in the blood varies rhythmically (1, 9) and because the administration of pharmacological doses of this compound is known to elevate tyrosine transaminase activity (10), it is possible that the steroid rhythm may make some contribution to the enzyme rhythm. Such a contribution, however, would normally be of minor significance compared with the effects of dietary protein: the amplitude of the transaminase rhythm is unaffected following hypophysectomy or adrenalectomy, so long as rats continue to have access to dietary protein (1).

LITERATURE CITED

1. Wurtman, R. J., and J. Axelrod 1967 Daily rhythmic changes in tyrosine transaminase activity of the rat liver. Proc. Nat. Acad. Sci. US, *57:* 1594.
2. Civen, M., R. Ulrich, B. M. Trimmer and C. R. Brown 1967 Circadian rhythms of liver enzymes and their relationship to enzyme induction. Science, *157:* 1563.
3. Shambaugh, G. E., D. A. Warner and W. R. Beisel 1967 Hormonal factors altering rhythmicity of tyrosine-alpha-ketoglutarate transaminase in rat liver. Endocrinology, *81:* 811.
4. Siegel, P. S., and H. L. Stackey 1947 The diurnal course of water and food intake in the normal, mature rat. J. Comp. Physiol. Psychol., *40:* 365.
5. Wurtman, R. J., W. J. Shoemaker and F. Larin 1968 Mechanism of the daily rhythm in hepatic tyrosine transaminase activity: role of dietary tryptophan. Proc. Nat. Acad. Sci. US, *59:* 800.
6. Diamondstone, T. I. 1966 Assay of tyrosine transaminase activity by conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde. Anal. Biochem., *16:* 395.
7. Snoddy, H. D., and R. W. Fuller 1968 Feeding schedule alteration of daily rhythm in tyrosine-alpha-ketoglutarate transaminase of rat liver. Science, *159:* 738.
8. Honova, E., S. A. Miller, R. A. Ehrenkranz and A. Woo 1968 Development of daily rhythm in tyrosine-α-ketoglutarate transaminase in neonatal rat liver. Science, *162:* 999.
9. Guillemin, R., W. E. Dear and R. A. Liebelt 1959 Nychthemeral variations in plasma-free corticosteroid levels of the rat. Proc. Soc. Exp. Biol. Med., *101:* 394.
10. Lin, E. C. C., and W. Knox 1957 Adaptation of the rat liver tyrosine-α-ketoglutarate transaminase. Biochim. Biophys. Acta, *26:* 85.

Pathological Defects in the Epiphyseal Cartilage of Zinc-deficient Chicks [1,2]

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ABSTRACT The objectives of this study were: 1) to compare the epiphyseal plates of the tibiotarsus and also the hock synovia of zinc-deficient and control chicks, 2) to gain information on the site of action of histamine and indomethacin, which were found previously to alleviate significantly the leg abnormalities caused by zinc deficiency, and 3) to compare the epiphyseal plates of zinc-deficient chicks with those from chicks deficient in manganese and in vitamin D. In the proliferating region of the epiphyseal plate of zinc-deficient chicks, the cartilage cells that were near blood vessels were normal, whereas cells remote from vessels were larger and rounder and were separated by more extracellular matrix. In these chicks the cells of the degenerating region of the epiphyseal plate did not degenerate normally unless they were near a blood vessel. In cross section these defects caused a characteristic and easily discernible "bull's eye" appearance around each blood vessel. This was not seen in tissue from manganese- or vitamin D-deficient chicks. Neither histamine nor indomethacin corrected the fundamental lesion due to zinc deficiency; however, the gross defect was alleviated. No hyperplasia or inflammation of the synovia due to zinc deficiency was observed.

The pathological defects of the hock joint of zinc-deficient chickens fed diets based on soybean protein have been described (1, 2). Grossly, the leg bones were shortened and thickened; the joint appeared swollen and twisted, and movement caused the chicken discomfort. In normal chicks the cells of the epiphyseal plate were arranged in long columns, and the epiphyseal-diaphyseal junction was a lengthy structure of cartilage tunneled by blood vessels. It was reported that in zinc-deficient chicks, the epiphyseal plate cartilage cells were arranged more randomly, the epiphyseal-diaphyseal junction was narrower, and penetration by blood vessel tunnels was markedly reduced.

Recent work in this laboratory has confirmed the observation that zinc-deficient chicks fed a diet based on soybean protein show a leg abnormality (3). Some synthetic chelating agents (4), histidine and histamine (5), and several antiarthritic and anti-inflammatory compounds [4] (6) have been found to alleviate the hock syndrome.

Because the epiphyseal plate is the site of bone elongation, and the zinc deficiency defect is characterized by altered bone growth and shape, this study was conducted in an attempt to compare further the structure of the epiphyseal plate in zinc-deficient and zinc-sufficient chicks fed diets based on isolated soybean protein. Moreover, the alleviating effects of numerous antiarthritic (i. e., anti-inflammatory) agents on this syndrome caused us to investigate the synovium of the joint as well as the bone for any similarities to arthritic syndromes in man. We hoped to find an objective assay for the severity of the syndrome and gain information on the site of action of histamine and of anti-inflammatory compounds in preventing obvious leg defects.

When pathological defects characteristic of zinc deficiency were found, they were compared with those of chicks deficient in manganese or vitamin D.

METHODS

The basal diet fed the chicks from 1 day of age is shown in table 1; it contained 13 to 16 ppm Zn on an air-dried basis. Zinc at 80 ppm was added as zinc oxide to the basal diet to form the control diet.

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[3] Supported by an NIH postdoctoral fellowship.
[4] Reimann, E. M. 1968 Ph.D. Thesis University of Wisconsin, Madison.

TABLE 1

Composition of the basal diet

Ingredient	%
Glucose monohydrate [1]	65.24
Soybean protein [2]	22.50
Corn oil	5.00
Choline chloride	0.20
Mineral mix [3]	6.00
Glycine	0.20
Methionine	0.50
Vitamin mix [4]	0.25
Vitamin A mix [5]	0.10
Alpha-tocopheryl acetate	0.01
Total	100.00

[1] Cerelose, Corn Products Company, New York.
[2] C-1 Assay Protein, Skidmore Enterprises, Cincinnati, Ohio.
[3] The mineral mix for 1 kg of diet contained: (in grams) $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.3; $Ca_3(PO_4)_2$, 14; $MgSO_4$, 2.44; NaCl, 8.9; $Fe_2(SO_4)_3 \cdot 6H_2O$, 0.28; $MnSO_4 \cdot H_2O$, 0.42; KI, 0.04; $CuSO_4 \cdot 5H_2O$, 0.02; and glucose, 2.56.
[4] The vitamin mix for 1 kg of diet contained: (in milligrams) thiamine·HCl, 100; niacin, 100; riboflavin, 16; Ca pantothenate, 20; vitamin B_{12} (0.001% triturate), 20; pyridoxine·HCl, 6; biotin, 0.6; folic acid, 4.0; inositol, 100; menadione, 5; vitamin D_3 conc (Vita Plus Corp., Madison, Wis.), 1 g (1500 ICU); and glucose monohydrate, 1.12 g.
[5] The vitamin A mix for 1 kg of diet contained: vitamin A conc (250,000 units/g, Chas. Pfizer and Company, New York), 1.0 g; and sucrose, 24.0 g to provide 10,000 IU/kg of diet.

Chicks fed a restricted amount of the zinc-supplemented diet were used as weight-paired controls. In the manganese-deficient diet, $MnSO_4$ was omitted from the mineral mix of the above control diet. The manganese-deficient and the control diets contained, on an air-dried basis, less than 4 and 140 ppm Mn, respectively. In some experiments histamine dihydrochloride (0.2% of the air-dried diet) or indomethacin (0.025% of air-dried diet) was added to both zinc-deficient and zinc-supplemented diets for comparison to their respective controls (6). The diet fed the vitamin D-deficient chicks has been described previously (7).

One-day-old chicks not segregated according to sex were placed in a stainless steel battery at 37 to 40°. The feed and water troughs were aluminum and distilled water was used throughout. Chicks were killed at 10 days, 3 weeks, and 4 weeks on experiment by decapitation. The hock joint was split longitudinally with a razor blade and fixed with 10% formalin plus 2% calcium acetate. In some cases the entire longitudinal section of the hock was decalcified either with nitric acid–70% ethanol (5:95 by volume) or with 10% EDTA, and embedded in paraffin before sectioning. At other times fixed epiphyseal plate tissue was frozen and cut on a freezing microtome.[5] No structural difference resulted from the different tissue preparation techniques. Routine hematoxylin and eosin staining was used. The epiphyseal tissue described in this study was from chicks on several different experiments. The descriptions apply to all chick tissue viewed which in each case represented at least three chicks per treatment group in each experiment.

RESULTS

The synovia of the zinc-deficient and control chickens appeared identical and had no apparent abnormalities. There was no evidence of hyperplasia or inflammation of the synovia as a result of zinc deficiency.

Because the bones of fowl do not have primary and secondary centers of ossification that correspond to those in mammalian bones, and because the structure of the analogous areas of the bone of fowls and mammals are different, there is controversy (8) about the use of the term epiphysis in fowl. Fusion of the tarsal bones with the tibia and the metatarsus on either side of the hock joint causes these areas to look much like classical mammalian epiphyses, and because of common usage of the term, epiphysis is used throughout this paper.

The chick epiphyseal plate can be divided into four regions: the proliferating region (composed of cells nearest the hyaline cartilage of the bone end and containing cells that appear flattened in longitudinal sections), the region of maturing cells (cells are larger and more rounded in longitudinal sections), the degenerating region (an area of cells that have lost varying amounts of their content) and the calcifying region. These last two regions overlap to some extent (fig. 1).

The entire epiphyseal plate of the fowl, unlike that of mammals, is penetrated by blood vessels. Even the proliferating region is penetrated by blood vessels that extend

[5] Lipshaw Manufacturing Inc., Detroit, Mich. 48210.

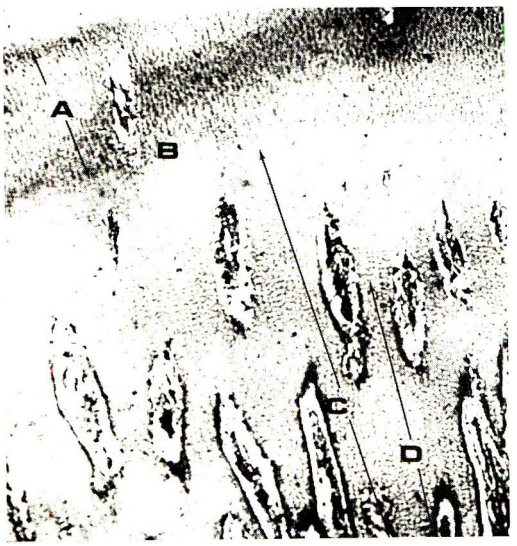

Fig. 1 Four-week-old, control (+ Zn) chick fed ad libitum. Longitudinal section of epiphyseal region of chick tibiotarsus A) proliferating region, B) maturing region, C) degenerating region and D) calcifying region. H & E. × 27.

from the marrow of the bone into the hyaline cartilage of the bone end. From the marrow cavity of the diaphysis additional blood vessels penetrate into the degenerating region and it is at the border between these later vessels and the degenerating cartilage cells that osteoblasts occur and calcification ensues. Therefore, cross sections through the proliferating and maturing regions have fewer blood vessels than sections through the degenerating areas further toward the diaphysis. In this paper the vessels that penetrate through the epiphyseal plate will be described as type 1 vessels and those that penetrate only into the degenerating area will be called type 2 vessels. Normal endochondral bone growth in the chick has been described by Wolbach and Hegsted (9).

The histopathological characteristics of the zinc deficiency syndrome in chicken hock epiphyseal plate are most easily seen in cross sections, although the defects are also apparent in longitudinal sections (fig. 2). Figures 3, 4, 5 and 6 show the appearance in cross section of the epiphyseal cartilage at different levels through the plate from 4-week-old chicks fed the zinc-supplemented diet ad libitum; comparative areas from zinc-deficient tissue are shown in figures 7, 8, 9 and 10. Except for a difference in width of the epiphysis, as discussed later, the zinc-supplemented chicks weight-paired by feed restriction to the deficient chicks appeared identical to

Fig. 2 Four-week-old zinc-deficient chick. Longitudinal section of epiphyseal region of chick tibiotarsus. Note the difference in amount of extracellular matrix between normal appearing areas (A), and abnormal areas (B). H & E. × 27.

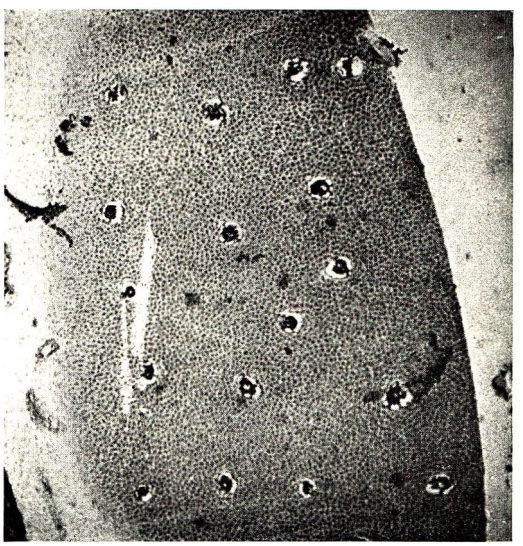

Fig. 3 Four-week-old, control (+ Zn) chick fed ad libitum. Cross section of the proliferating region of the epiphysis. Note the regularity of cell arrangement. H & E. × 27.

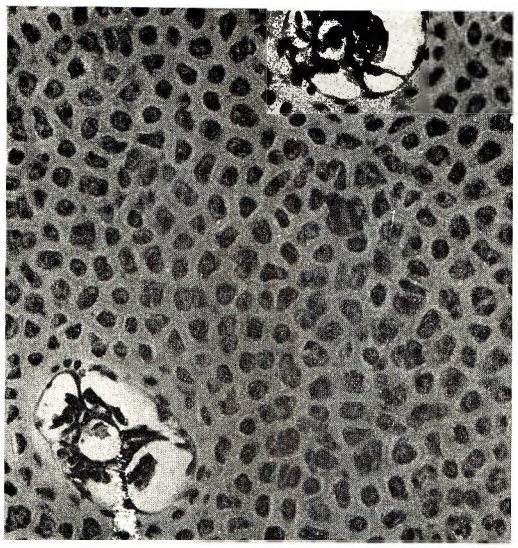

Fig. 4 Four-week-old, control (+ Zn) chick fed ad libitum. Cross section of the proliferating region of the epiphysis. Note the regularity of cell arrangement and width of extracellular matrix which fills the area between cells. H & E. × 173.

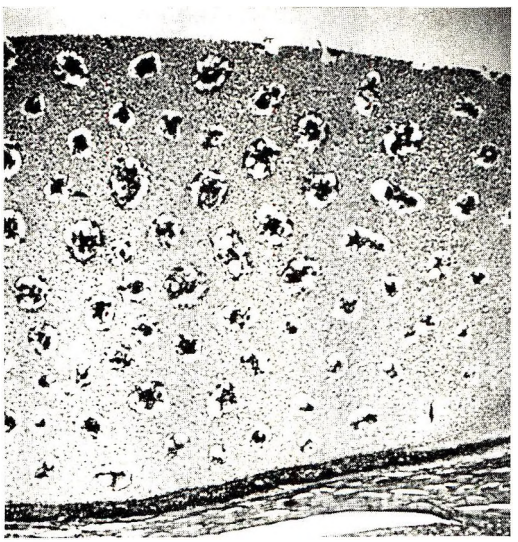

Fig. 5 Four-week-old, control (+ Zn) chick fed ad libitum. Cross section of the degenerating region of the epiphysis. Note the regularity of cell arrangement. H & E. × 27.

the zinc-supplemented chicks fed ad libitum. Thus, the differences ascribed to zinc deficiency were not caused by inanition. In the proliferating region of the zinc-deficient chick epiphyseal cartilage (figs. 7 and 8), only the cells near the blood vessels appeared normal, although there was a denser population of cells near the vessels in zinc-deficient chicks. In contrast, more extracellular matrix material and

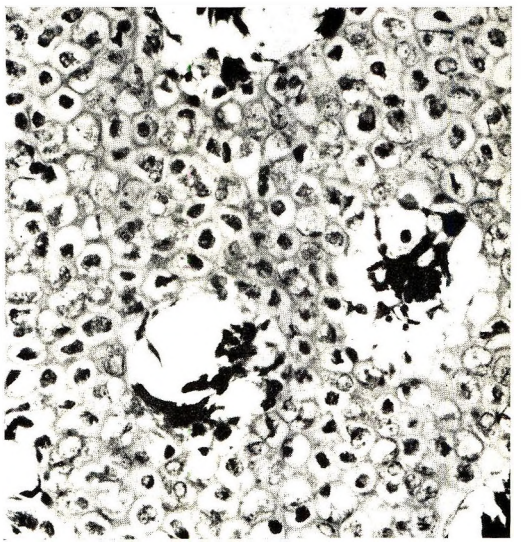

Fig. 6 Four-week-old, control (+ Zn) chick fed ad libitum. Cross section of the degenerating region of the epiphysis. Note that most of the cells are at about the same stage of degeneration and that the extracellular matrix is evenly distributed. H & E. × 173.

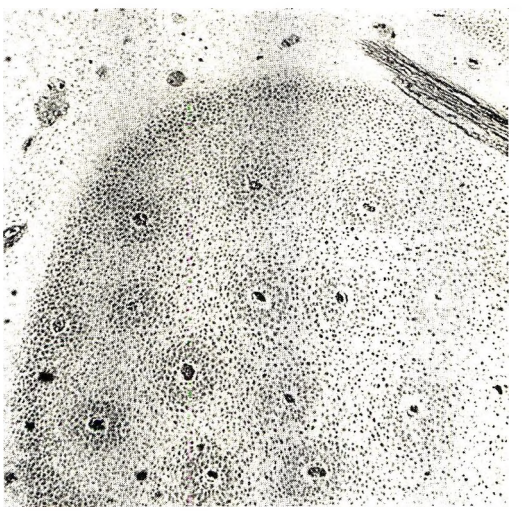

Fig. 7 Four-week-old zinc-deficient chick. Cross section of the proliferating region of the epiphysis. Note the unevenness of cell arrangement that produces a "bull's eye" effect around the blood vessels, also note that there are as many vessels as in figure 3. H and E. × 27.

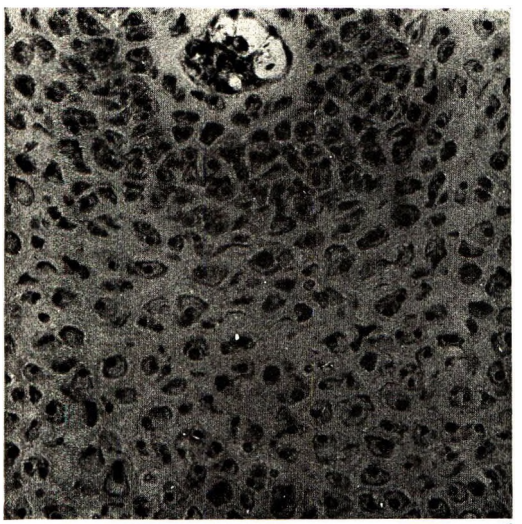

Fig. 8 Four-week-old zinc-deficient chick. Cross section of the proliferating region of the epiphysis. Note that the cells near the blood vessel are more tightly packed and there is more extracellular matrix between the cells remote from the blood vessel. H & E. × 173.

fewer cells were seen in areas more remote from the blood vessels of zinc-deficient chicks. These remote cells were slightly larger and were more rounded, possibly because of lack of intimate contact with neighboring cells. These defects in the zinc-deficient chicks caused the characteristic and easily discernible "bull's eye" appearance about each blood vessel. Longitudinal sections through the proliferating area (fig. 2) showed that the 5 to 10 cells nearest the hyaline cartilage of the bone end and also the cells near blood vessels were flattened and appeared normal. Areas which were near neither the hyaline cartilage of the bone end nor the penetrating blood vessels had more matrix, and the cells instead of being uniformly flattened and in regular columns as in normal bone, had irregular shapes and more random locations.

Cross sections through the degenerating region of the epiphysis (figs. 9 and 10) of zinc-deficient chicks showed defects similar to those described above for the proliferating region. Only cells near type 1 vessels were degenerating uniformly, whereas in all other areas the cells were in various stages of maturation and degeneration with very few having the typical degenerated appearance shown in figure 6. All three age groups tested showed similar defects but they were more pronounced at 3 and 4 weeks than at 10 days.

The area described earlier, in which degenerating cartilage cells and the zone of calcification overlap, is often called the metaphysis, and in control chicks it is extensive (fig. 1). When seen in longitudinal section, long columns of degenerating cells are penetrated by blood vessel tunnels, along the edge of which are osteoblasts and areas of calcification. In zinc-deficient chicks, this area was much narrower than in weight-paired controls and relatively few degenerating cells were seen on the diaphyseal side of the most proximal calcification (fig. 2).

In some longitudinal sections, it appeared that neither type 1 nor type 2 blood vessels penetrated the epiphyseal plate from zinc-deficient animals as abundantly as in control tissue. This has been reported previously (1). The excess of extracellular matrix and nonuniform degeneration of

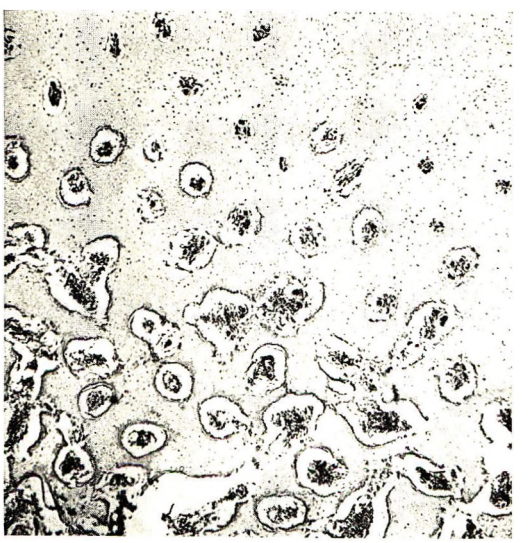

Fig. 9 Four-week-old zinc-deficient chick. Cross section of the epiphysis. Because of the convex shape of the epiphyseal plate, all regions appear in this figure: proliferating region in upper right; calcifying region in middle left and all of the lower part; and degenerating region between. Note that certain blood vessels (type 1) have a "bull's eye" of normally degenerated cells, whereas others (type 2) do not. The cells remote from these vessels are in various stages of degeneration. H & E. × 27.

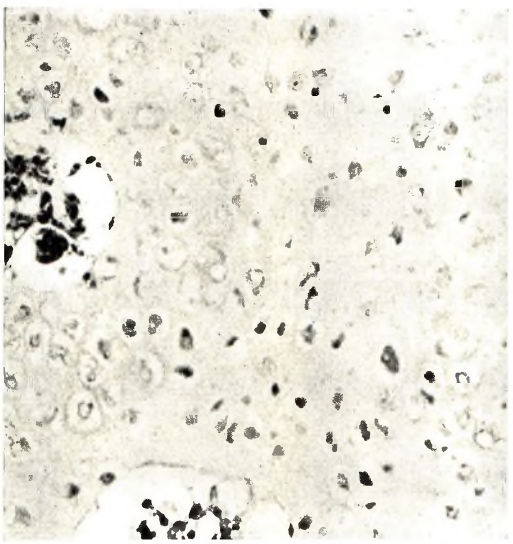

Fig. 10 Four-week-old zinc-deficient chick. Cross section of the degenerating region of the epiphysis. The upper left blood vessel is of type 1 and has around it normally degenerated cells. There is another type 1 vessel just off the picture at lower right and the edge of a normally degenerated "bull's eye" of cells can be seen. The lower left vessel is of type 2; note the area with excess extracellular matrix and cells in various stages of degeneration extending through the middle right part of the picture. H & E. × 173.

zinc-deficient tissue would make this appear reasonable. The more critical evaluation for blood vessels at any given cross sectional level, however, showed that there were approximately equal numbers of vessels per unit area in both zinc-deficient and control tissue.

The epiphyseal plates of control chicks weight-paired to the zinc-deficient chicks by feed retriction were indistinguishable from those of full-fed controls, except in total width of the epiphyseal plate. When growth rate was faster, the epiphysis was wider as has been reported previously.[6] Between zinc-deficient chicks and weight-paired controls, no difference occurred in the width of the epiphyseal plate from the proliferating region to the early degenerating cells. As discussed previously, however, the metaphysis was much narrower in the zinc-deficient chicks.

The figures shown are all of epiphyses from 4-week-old chicks; however, the defects described were present to nearly the same degree at 3 weeks and were less pronounced but definite at 10 days of age.

The epiphyseal tissue from manganese-deficient chicks showed the disorganization described previously (10). The cells of the epiphyseal plates of vitamin D-deficient chicks were irregularly arranged compared with the controls. Contrary to the zinc deficiency syndrome, in neither manganese-deficient or vitamin D-deficient chicks did proximity to blood vessels have any effect.

Neither histamine nor indomethacin had any readily discernible effect on the epiphyseal morphologic syndrome caused by zinc deficiency although they did alleviate, in part, the gross leg defect and lameness of the chickens as observed previously (6).

DISCUSSION

The simplest explanation for the pathologic defects of the epiphyseal plate due to zinc deficiency is that the cells that are nearer sources of nutrition (i.e., blood vessels) obtain enough of the limiting nutrient, zinc, and are normal, whereas cells more remote from the nutrient sources develop abnormally because of lack of zinc. Another possibility is that zinc (e.g., a zinc metalloenzyme) is necessary for the transport from the blood vessels to the cartilagenous cells of a substance or substances that are essential for normal epiphyseal cell maturation and degeneration.

In chicks deficient in manganese or vitamin D, no relationship of cell defect to proximity of blood vessels occurred.

There is no obvious explanation for the fact that the metaphyseal region is much different in the bones of control and zinc-deficient chicks. It may be that normal calcification, however, which occurs simultaneously around the entire circumference of blood vessels penetrating the degenerating region of normal chickens, protects from resorption the degenerating cells that remain. In zinc-deficient tissue, gaps in calcification on sides of the vessels because of delayed cell development might fail to protect from resorption the remaining degenerated cells. This could result in the picture seen in which the metaphysis

[6] Fahmy, A. 1956 An experimental study of some factors affecting bone growth. Ph.D. Thesis, Institute of Orthopaedics, University of London, England.

has no long columns of degenerating cells deep into the bone spongiosa, as it does in normal bone.

Wolbach and Hegsted suggested (10) that the twisting of the hock joint in perosis is due to weakening of the metaphyseal region. The histologic picture of this region is similar in perosis and zinc deficiency, and their suggestion seems logical though still unconfirmed. One further fact that lends credence to this idea is that heavier chicks usually had hocks that were more twisted, as if a physical weakness might be the cause. Body weight, however, had no effect on the histologic syndrome.

The increased amount of extracellular matrix material seen in areas not near vessels may account for some of the thickening or swelling of the bones, but this is also not proved. Swelling could also be caused by continued proliferation, but delayed maturation, of cells, and thus piling up of cells in areas remote from the blood vessels.

The mechanism whereby histamine and indomethacin help to alleviate the lameness associated with this syndrome was not indicated in this study. It can be said, however, that these agents do not correct the fundamental lesion due to zinc deficiency, but in some way moderate the gross defect. There was no sign of inflammation or hyperplasia of the synovia of even the untreated zinc-deficient chicks, so the synovial membrane is probably not the tissue beneficially affected by these agents.

This investigation indicates that the histopathologic defects in chick bone caused by zinc deficiency are distinctly different and recognizable from those caused by manganese deficiency.

ACKNOWLEDGMENTS

We thank Dr. H. F. DeLuca for the vitamin D-deficient chicks and E. C. Faltin for technical assistance.

LITERATURE CITED

1. Young, R. J., H. M. Edwards, Jr. and M. B. Gillis 1958 Studies on zinc in poultry nutrition. 2. Zinc requirement and deficiency symptoms of chicks. Poultry Sci., *37:* 1100.
2. O'Dell, B. L., P. M. Newberne and J. E. Savage 1958 Significance of dietary zinc for the growing chicken. J. Nutr., *65:* 503.
3. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1966 Effect of dietary amino acid source on the zinc-deficiency syndrome in the chick. J. Nutr., *89:* 24.
4. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1966 Effect of some dietary synthetic and natural chelating agents on the zinc-deficiency syndrome in the chick. J. Nutr., *89:* 35.
5. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1967 Effect of histamine, histidine, and some related compounds on the zinc-deficient chick. Proc. Soc. Exp. Biol. Med., *124:* 1106.
6. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1968 Alleviation of the leg abnormality in zinc-deficient chicks by histamine and by various anti-arthritic agents. J. Nutr., *94:* 527.
7. Imrie, M. H., P. F. Neville, A. W. Snellgrove and H. F. DeLuca 1967 Metabolism of vitamin D_2 and vitamin D_3 in the rachitic chick. Arch. Biochem. Biophys., *120:* 525.
8. Church, L. E., and L. C. Johnson 1964 Growth of long bones in the chicken. Amer. J. Anat., *114:* 521.
9. Wolbach, S. B., and D. M. Hegsted 1952 Endochondral bone growth in the chick. Amer. Med. Assoc. Arch. Pathol., *54:* 1.
10. Wolbach, S. B., and D. M. Hegsted 1953 Epiphyseal cartilage in choline and manganese deficiencies in the chick. Amer. Med. Assoc. Arch. Pathol., 56: 437.

Histochemical Studies of Alkaline Phosphatase in Epiphyseal Cartilage of Normal and Zinc-deficient Chicks [1,2]

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ABSTRACT The object of this study was to compare histochemically the distribution of alkaline phosphatase, mucopolysaccharides and collagen in the epiphyseal plates of normal chicks, zinc-deficient chicks and zinc-deficient chicks fed histamine or indomethacin. A diet based on isolated soybean protein was used and manganese-deficient chicks were studied for comparison. Alkaline phosphatase in normal chick epiphysis was found in the proliferating cells near the maturing region, in maturing cells, and in degenerating cells which contained remnants of cellular elements. Little or no alkaline phosphatase was present in early proliferating cells or in fully degenerated cells, but it was again found in calcifying areas. The epiphyseal plate cells of zinc-deficient chicks showed this normal progression of alkaline phosphatase activity *only* if they were near blood vessels or in areas of calcification, where proximity to blood vessels had no effect and normal alkaline phosphatase activity was found. In the deficient chick, cells remote from blood vessels were much delayed in their development of alkaline phosphatase. No difference in distribution of mucopolysaccharides or collagen was found. The epiphysis from zinc-deficient chicks fed histamine or indomethacin showed the same distribution of alkaline phosphatase as did zinc-deficient tissue even though these agents grossly moderated the leg defect. Manganese deficiency did not affect the distribution of alkaline phosphatase in chick epiphyseal tissue. Alkaline phosphatase in noncalcifying chick epiphyseal plate tissue appeared to be necessary for normal cell maturation and degeneration, processes which were defective in the zinc-deficient chicks. This and other theories about alkaline phosphatase function are discussed.

Theories on the mechanism of calcification almost invariably include a role for alkaline phosphatases (1–3). In several animal species, alkaline phosphatases are known to be zinc metalloenzymes (4, 5). Zinc-deficient turkey poults have been shown to develop a characteristic hock disorder and have decreased alkaline phosphatase activity in the bone ends (6).

Henrichsen has studied alkaline phosphatase in a) fibroblasts and osteoblasts in tissue culture (7, 8), b) in the calcification of tuberculous lymph nodes (9), and c) in chick embryonic bone (10). He has presented evidence that the role of alkaline phosphatase is often a degenerative one. In our morphological studies (11) the epiphyseal plate cells from zinc-deficient chicks appeared to mature and degenerate abnormally.

The cells of the epiphyseal plate are active in the secretion of extracellular matrix containing collagen and mucopolysaccharides. Many investigators have linked high alkaline phosphatase activity to extracellular matrix secretion (12–15), and some specifically to collagen synthesis (16, 17) and mucopolysaccharide synthesis (18).

Therefore, it seemed logical to study the histochemical distribution of alkaline phosphatase, mucopolysaccharides and collagen in zinc-deficient and control chick epiphyseal plate tissue.

Recent work in this laboratory has shown that histamine and several antiarthritic compounds help to alleviate the leg abnormality of zinc-deficient chicks [4] (19). Tissues from chickens that were zinc-deficient but had been fed histamine or indomethacin were included in the hope

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[2] Supported in part by Public Health Service Grant no. AM-05606 from the National Institute of Arthritis and Metabolic Diseases.
[3] Supported by an NIH postdoctoral fellowship.
[4] Reimann, E. M. 1968 Ph.D. Thesis University of Wisconsin, Madison.

that the site of action of these agents in the relief of lameness might be found.

METHODS

The diets and experimental conditions were as described previously (11). As in the previous study, at least three chicks were studied carefully in any treatment group and any effects noted in this paper were consistent in all chicks observed. To be certain that any effects of zinc deficiency were not the result of restricted growth, zinc-deficient chicks were compared with controls fed ad libitum as well as to weight-paired controls which were restricted in feed intake.

One-day-old chicks were fed the experimental diets and were killed at 4 weeks. The hock joint was split with a razor and the epiphyseal plate region was removed. Fixation was in 10% formalin plus 2% calcium acetate for 2 to 4 hours. Tissues were rinsed and left overnight in 0.1 M PO_4 buffer at pH 7.4 to which sucrose had been added (to make 0.135 M) to bring the osmolality to about 425 milliosmols/kg. Routinely, the tissues were then embedded in paraffin prior to histochemical staining; however, alkaline phosphatase staining was preceded by freezing the tissue upon removal from the buffer and cutting with a freezing microtome.[5]

The techniques used for the various histochemical tests were: alkaline phosphatase (20), mucopolysaccharides (21–23) and collagen (24).

RESULTS

The various regions of chick epiphyseal plate were described in a previous publication (11).

Figures 1, 2 and 3 illustrate alkaline phosphatase distribution in the epiphyseal plate of normal chicks fed ad libitum the diet supplemented with zinc (80 ppm). However, cross sections from zinc-supplemented chicks weight-paired by feed restriction to the deficient chicks were indistinguishable from the controls fed ad libitum. In the normal chick epiphyseal plate, alkaline phosphatase activity occurred in the regions of the proliferating cells nearest the mature cartilage cells (fig. 1), but not in the proliferating cells nearest the hyaline cartilage. Enzyme activity was also high in the mature cells (fig. 2) and in degenerating cells as long as cell remnants remained (fig. 2). In areas of maturing and early degenerating cells the cellular elements were stained, but extracellular matrix material stained little, if at all. Fully degenerated cells had essentially no alkaline phosphatase activity. Thus, as the cells developed and differentiated into mature and then degenerating cells, there was a profound increase in alkaline phosphatase activity followed by a sharp decrease. There was also alkaline phosphatase activity in areas where calcification was taking place (fig. 3); this staining was extracellular as well as in the cell.

Figures 4, 5 and 6 illustrate the alkaline phosphatase distribution in the epiphyseal plate of zinc-deficient chicks. Only if they were near blood vessels did the cells of the epiphyseal plate of zinc-deficient chicks show the normal progression of staining for alkaline phosphatase as the cells matured and degenerated (see above) (figs.

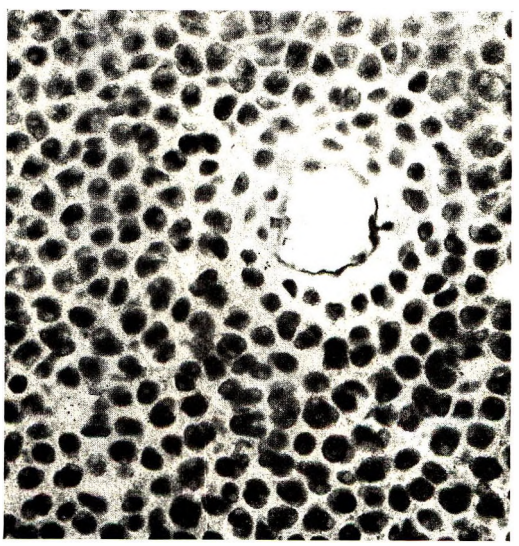

Fig. 1 Control (+ Zn) chick fed ad libitum. Cross section of the proliferating region of the epiphysis near the mature region. Note that the relation to blood vessels had no effect on intensity of cellular staining and that the extracellular matrix was not stained. The azo-coupled alkaline phosphatase stain was rose colored but appears black in the figures. Alkaline phosphatase stain. × 173.

[5] Lipshaw Manufacturing Inc., Detroit, Mich. 48210.

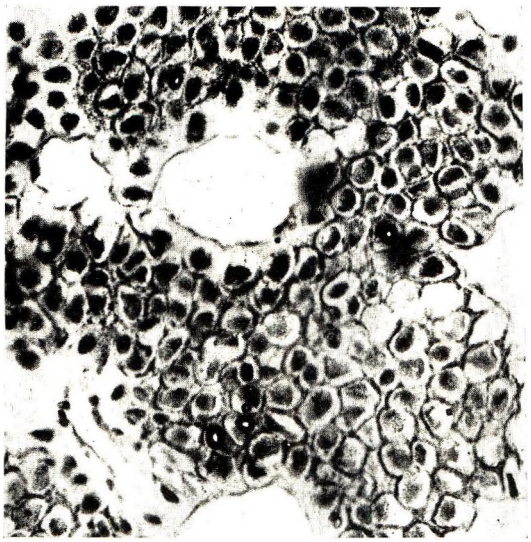

Fig. 2 Control (+ Zn) chick fed ad libitum. Cross section of the degenerating region of the epiphysis; the upper left is nearest the mature region and the lower right is nearest the calcifying region. Note that all cellular elements present stained and that the relation to the blood vessel had no effect on the intensity. Extracellular matrix was not stained. Alkaline phosphatase stain. × 173.

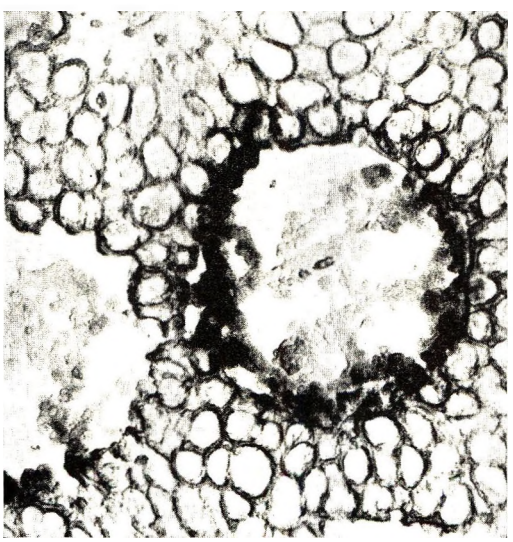

Fig. 3 Control (+ Zn) chick fed ad libitum. Cross section of the calcifying region of the epiphysis. Note that only the calcifying area just at the edge of the vessel is stained and that the stain is both extra- and intracellular. Alkaline phosphatase stain. × 173.

4 and 5). Cells remote from type 1 blood vessels (which completely penetrate through the epiphyseal plate) (11) did not stain for alkaline phosphatase until they

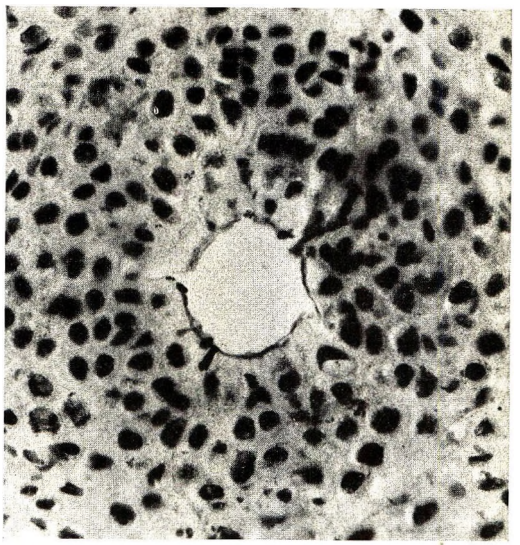

Fig. 4 Zinc-deficient chick. Cross section of the proliferating region of the epiphysis near the mature region. Note that the cells near the blood vessel stained more darkly and that extracellular matrix was not stained. Compare with figure 1. Alkaline phosphatase stain. × 173.

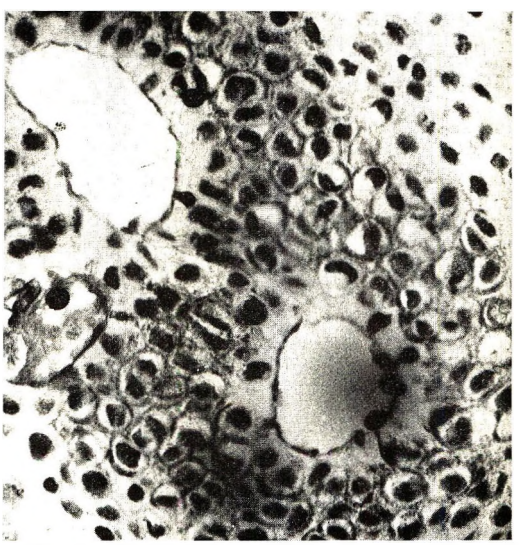

Fig. 5 Zinc-deficient chick. Cross section of the degenerating region of the epiphysis. Note that the cells nearest the blood vessel stained more darkly and that the extracellular matrix was not stained. Compare with figure 2. Alkaline phosphatase stain. × 173.

approached the normally degenerating region, in which invading type 2 blood vessels had penetrated into their immediate proximity; after this they showed alkaline phosphatase activity and then seemingly degenerated (fig. 5). It appeared that the cells away from the type 1 blood vessels were greatly delayed in their differentiation and development until they were provided nutrients from the invading type 2 vessels at which time they developed, but in an apparently erratic and possibly incomplete manner. In zinc-deficient tissue, calcifying areas stained for alkaline phosphatase like controls (fig. 6).

Epiphyseal plate tissue from zinc-deficient chicks fed either indomethacin or histamine could not be distinguished from their control zinc-deficient chick tissue by any staining methods tried, despite the fact that these agents grossly moderated the leg defect as observed previously (19).

Epiphyseal plate tissue from manganese-deficient chicks stained for alkaline phosphatase activity as did normal chick tissue (figs. 7, 8 and 9). In manganese-deficient chicks that have perosis, bone alkaline phosphatase activity is lowered (25, 26). In this case the alkaline phosphatase measured was probably primarily located in areas of active calcification, and because these areas are more extensive in normal than in perotic chickens, the results are reasonable. The data reported here indi-

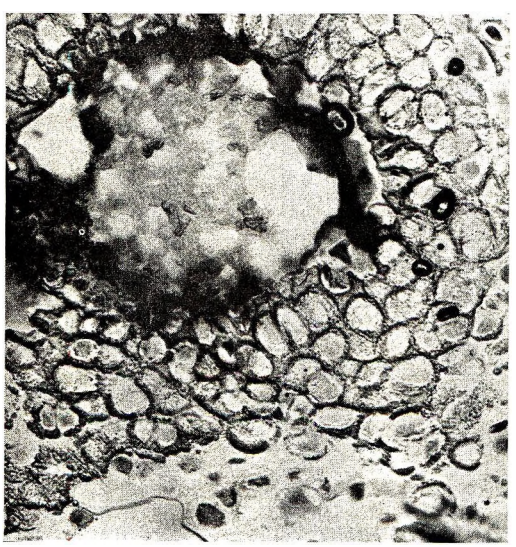

Fig. 6 Zinc-deficient chick. Cross section of the calcifying region of the epiphysis. Note that there is no difference in staining of the calcifying region from figure 3. At the bottom of the figure are cells that have not degenerated completely and whose remnants stained positively for alkaline phosphatase. Alkaline phosphatase stain. × 173.

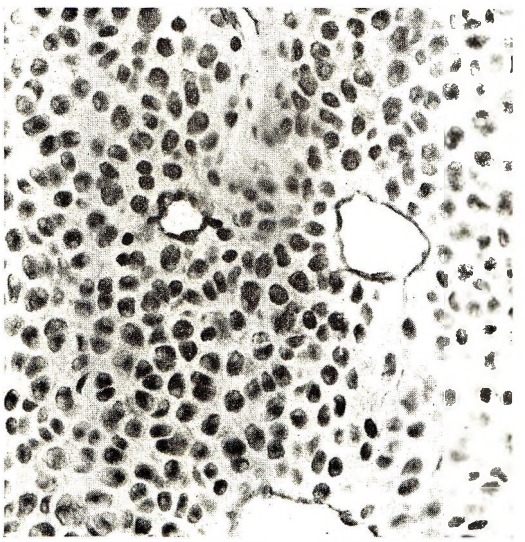

Fig. 7 Manganese-deficient chick. Proliferating region of the epiphysis. Note similarity to figure 1. Alkaline phosphatase stain. × 173.

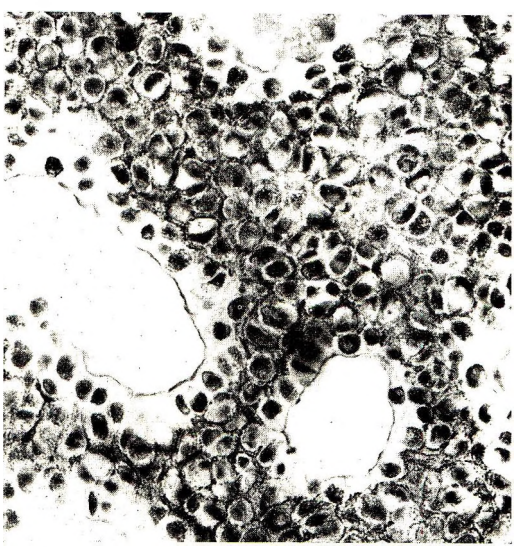

Fig. 8 Manganese-deficient chick. Degenerating region of the epiphysis. Note similarity to figure 2. Alkaline phosphatase stain. × 173.

Fig. 9 Manganese-deficient chick. Degenerating region of the epiphysis. Note similarity to figure 3. Alkaline phosphatase stain. × 173.

cate that the alkaline phosphatase of noncalcifying epiphyseal plate is not affected by manganese deficiency, but is markedly affected by zinc deficiency.

Staining for either mucopolysaccharides by several methods or for collagen failed to reveal any differences between zinc-deficient and control tissue except in the absolute amount of extracellular matrix, which in zinc deficiency was increased around the cells remote from type 1 vessels as described previously (11).

DISCUSSION

The suggestion has been made that one of the functions of alkaline phosphatase is in collagen synthesis. This has been based on a) the high activity in regenerating tendon (12); b) the high activity in healing skin wounds (14); and c) the observation that vesicles rich in hydroxyproline and alkaline phosphatase are located near fibrils of chick bone cells that presumably, when extruded, contribute to collagen fibers (17). There are also many reports of high alkaline phosphatase activity in tissues that excrete extracellular matrix. Moog and Wenger (18) have shown histochemically that, in embryonic chick bone, alkaline phosphatase activity was high in the same areas that stain for mucopolysaccharides.

The data reported here indicate that in cartilage cells of the proliferating, maturing, and early degenerating regions of the epiphyseal plate of zinc-deficient chicks, alkaline phosphatase was not necessary for secretion of extracellular matrix. The abnormal cells away from the blood vessels, although low in alkaline phosphatase, were associated with especially abundant extracellular collagen and mucopolysaccharides. Possibly the matrix was abnormal in these areas but nothing from this study suggested this. Extensive mucopolysaccharide histochemistry showed no difference except for the increased amount of matrix away from blood vessels in zinc-deficient tissue. The same was true for collagen staining. Electron-microscopic studies [6] also failed to reveal differences in amount of collagen, mucopolysaccharide or ratios of one to the other in the areas mentioned above. The results do not rule out the possibility that the matrix in zinc-deficient tissue not near blood vessels is abnormal in some way and ultrastructural differences may yet be found. From the present results, however, it appears that if alkaline phosphatase is to be assigned a role in matrix formation in noncalcifying chick epiphyseal plate cartilage, it would be more logical that its role is in control of synthesis rather than one of absolute necessity for synthesis.

It appears that the function of alkaline phosphatase in the region of active calcification is distinct from its function in the proliferating, maturing, and early degenerating areas of epiphyseal plate cartilage of chicks. In this study, no difference between zinc-deficient and control tissues was seen in either intensity of staining for alkaline phosphatase or distribution of areas stained in calcifying regions. Also, the alkaline phosphatase activity in the noncalcifying cartilage was predominately, if not entirely, in the cell, whereas in calcifying regions the matrix stained deeply. Starcher and Kratzer (6) found that in turkey poults the level of alkaline phosphatase activity in the bone ends was lowered much more due to zinc deficiency than

[6] Westmoreland, N. P., and W. G. Hoekstra, unpublished results.

was the level in the diaphysis. They stated that this was possibly because their poults were not fed the deficient diet until they were 5-days old and some diaphyseal alkaline phosphatase activity remained from the early days of life. From the present study, a more likely explanation of their results is that the alkaline phosphatase associated with calcification is little affected by zinc deficiency, whereas the enzyme associated with the developing epiphyseal plate cartilage is markedly affected. Also, the cells of zinc-deficient epiphyseal plate remote from blood vessels developed alkaline phosphatase as they approached the diaphysis and the cartilage became invaded by more blood vessels. It is clear from this study that the effect of zinc deficiency on bone alkaline phosphatase is highly dependent on the exact site within bone from which the sample is obtained.

In studies on the developmental pattern and specificities of alkaline phosphatases in embryonic chick limb, it was noted (27) that bone alkaline phosphatase was different from the enzyme found in muscle and the integument. It would be of interest to ascertain if a similar difference exists between the enzyme seen in calcifying regions and that seen in noncalcifying epiphyseal cartilage.

From the present study, we concur with the degenerative role that Henrichsen (7–10) suggested for alkaline phosphatase in certain cells. It appears to function in the maturation and degeneration of chick epiphyseal plate cartilage. Cells in zinc-deficient epiphyseal plate cartilage that were not near blood vessels were abnormal morphologically, failed to degenerate normally, and histologically showed much less alkaline phosphatase activity. Later, when blood vessels invaded their immediate area they did stain for alkaline phosphatase and then degenerated. Zinc probably does not serve a simple prosthetic function, because enough zinc to serve such a purpose was likely present in the incubation medium and any apoenzyme would have been activated. The simplest explanation is that the cells need zinc before they can synthesize this alkaline phosphatase, and cells remote from the blood vessels may not have received enough zinc for this purpose. This is not proved, however, and zinc may be involved in the transport to these cells of some other substance necessary for normal differentiation characterized by appearance of alkaline phosphatase (11).

The fact that normal proliferating cells from epiphyseal plate cartilage began to stain for alkaline phosphatase only as they approached the maturing region, coupled with a consideration of the maturation process as the beginning of degeneration, also supports the theoretical degenerative role for alkaline phosphatase in noncalcifying chick epiphyseal cartilage. Moreover, the peak of alkaline phosphatase activity has been related to cartilage cell degeneration in a study on osteogenesis in vitro using embryonic chick femora (28).

LITERATURE CITED

1. Robison, R. 1923 The possible significance of hexosephosphoric esters in ossification. Biochem. J., *17:* 286.
2. Siffert, R. S. 1951 The role of alkaline phosphatase in osteogenesis. J. Exp. Med., *93:* 415.
3. Fleish, H., and W. F. Neuman 1961 Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. Amer. J. Physiol., *200:* 1296.
4. Mathies, J. C. 1958 Preparation and properties of highly purified alkaline phosphatase from swine kidneys. J. Biol. Chem., *233:* 1121.
5. Trubowitz, S., D. Feldman, S. W. Morgenstern and V. M. Hunt 1961 The isolation, purification and some properties of the alkaline phosphatase of human leucocytes. Biochem. J., *80:* 369.
6. Starcher, B., and F. H. Kratzer 1963 Effect of zinc on bone alkaline phosphatase in turkey poults. J. Nutr., *79:* 18.
7. Henrichsen, E. 1956 Alkaline phosphatase in osteoblasts and fibroblasts cultivated *in vitro*. Exp. Cell Res., *11:* 115.
8. Henrichsen, E. 1956 Alkaline phosphatase and the calcification in tissue cultures. Exp. Cell Res., *11:* 403.
9. Henrichsen, E. 1956 Alkaline phosphatase and calcification in tuberculous lymph nodes. Exp. Cell Res., *11:* 511.
10. Henrichsen, E. 1958 Bone formation and calcification in cartilage. Acta Orthop. Scand., *27:* 173.
11. Westmoreland, N., and W. G. Hoekstra 1969 Pathological defects in the epiphyseal cartilage of zinc-deficient chicks. J. Nutr., *98:* 76–82.
12. Buck, R. C. 1953 Regeneration of tendon. J. Pathol. Bacteriol., *66:* 1.
13. Moog, F. 1944 Localization of alkaline and acid phosphatases in the early embryogenesis of the chick. Biol. Bull., *86:* 51.

14. Fell, H. B., and J. F. Danielli 1943 The enzymes of healing wounds. I. The distributions of alkaline phosphomonoesterase in experimental wounds and burns in the rat. Brit. J. Exp. Pathol., *24:* 196.
15. Prichard, J. J. 1952 A cytological and histochemical study of bone and cartilage formation in the rat. J. Anat., *86:* 259.
16. Fitton Jackson, S. 1954 The formation of connective and skeletal tissues. Proc. Roy. Soc. (London) Series B, *142:* 536.
17. Fitton Jackson, S., and R. H. Smith 1955 Fibrogenesis of connective and skeletal tissues in the embryonic fowl. Symp. Soc. Exp. Biol., *9:* 89.
18. Moog, F., and E. L. Wenger 1952 The occurrence of a neutral mucopolysaccharide at sites of high alkaline phosphatase activity. Amer. J. Anat., *90:* 339.
19. Nielsen, F. H., M. L. Sunde and W. G. Hoekstra 1968 Alleviation of the leg abnormality in zinc-deficient chicks by histamine and by various anti-arthritic agents. J. Nutr., *94:* 527.
20. Barka, T., and P. J. Anderson 1963 Phosphatase alkaline. 1. Simultaneous coupling azo dye method. Histochemistry. Harper & Row Publishers, New York, p. 231.
21. Lev, R., and S. S. Spicer 1964 Specific staining of sulfate groups with alcian blue at low pH. J. Histochem. Cytochem., *12:* 309.
22. Wetzel, M. G., B. K. Wetzel and S. S. Spicer 1966 Ultrastructural localization of acid mucosubstances in the mouse colon with iron-containing stains. J. Cell Biol., *30:* 299.
23. Spicer, S. S., R. G. Horn and T. J. Leppi 1967 Histochemistry of connective tissue mucopolysaccharides. In: The Connective Tissue, eds., B. M. Wagner and D. E. Smith. Int. Acad. Pathol. Monogr. no. 7. Williams & Wilkins Company, Baltimore, p. 251.
24. Humason, G. L. 1962 Animal Tissue Techniques. W. H. Freeman and Company, San Francisco and London, p. 171.
25. Wiese, A. C., B. C. Johnson, C. A. Elvehjem, E. B. Hart and J. G. Halpin 1939 A study of blood and bone phosphatase in chick perosis. J. Biol. Chem., *127:* 411.
26. Wiese, A. C., G. H. Benham, C. A. Elvehjem and E. B. Hart 1941 Further bone phosphatase studies in chick perosis. Poultry Sci., *20:* 255.
27. McWhinnie, D. J., and J. W. Saunders, Jr. 1966 Developmental patterns and specificities of alkaline phosphatase in the embryonic chick limb. Develop. Biol., *14:* 169.
28. Teaford, M. E., and A. A. White 1964 Alkaline phosphatase and osteogenesis *in vitro*. Proc. Soc. Exp. Biol. Med., *117:* 541.

Effect of Dietary Protein Levels on the Toxicity and Metabolism of Heptachlor [1]

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ABSTRACT The purpose of this study was to show the effects of dietary protein levels on the toxicity and metabolism of heptachlor. Male weanling rats fed either a 5, 20 or 40% casein diet for 10 days were given heptachlor (a chlorinated hydrocarbon insecticide) intraperitoneally for LD_{50} determinations. Rats receiving the 5% casein diets exhibited a threefold tolerance to heptachlor, compared with their pair-fed mates, due to their inability to form the toxic metabolite, heptachlor epoxide. The epoxide itself was more toxic than heptachlor when administered intraperitoneally, and its toxicity was not influenced by the dietary regimen of the rats. Metabolism studies revealed that rats fed the 20 and 40% casein diets produced more of the epoxide at a faster rate than those fed the 5% casein diet. Thus, populations on low protein diets may fare better than their well-fed counterparts when exposed to heptachlor and possibly other enzymatically activated toxicants.

Pesticides, particularly the chlorinated hydrocarbons, retain their bioactivity for long periods of time and represent a potential hazard to nontarget organisms. These compounds are employed for crop protection, storage and transport of food and frequently are used in areas where populations exist on nutritionally inadequate diets, thus posing additional problems.

That the interactions of nutrients and pesticide toxicity have not been extensively studied is evident. Phillips (1) showed that nutrient-pesticide interactions occur by observing that 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) in the diet of rats decreased liver stores of vitamin A. Dietary dieldrin also reduced liver vitamin A (2). Krishnamurthy et al. (3) indicated that rats fed poor rice diets were more susceptible to dieldrin than were rats fed nutritionally adequate diets. Stickel et al. (4) reported that body condition, as reflected by body weight, could influence DDT and heptachlor toxicity in woodcocks. Those birds which lost weight while adapting to captive conditions showed higher mortality than woodcocks which did not suffer weight loss. Donaldson et al. (5) placed chicks on DDT-supplemented diets for 4 weeks and the birds were either starved or fed DDT-free diets for 96 hours. The starved birds mobilized DDT stores more rapidly as evidenced by higher DDT levels in the blood. Boyd and DeCastro (6) concluded that low protein diets did not significantly alter DDT toxicity in the rat. Similar studies with lindane (7) revealed that rats fed cachectic diets were twice as susceptible to lindane poisoning as were controls.

Heptachlor (1,4,5,6,7,8,8-heptachloro-3a, 4, 7, 7a-tetrahydro-4,7-methanoindene), a chlorinated hydrocarbon insecticide, is enzymatically converted to its more toxic metabolite, heptachlor epoxide, by rat liver microsomes (8). The enzymatic nature of this reaction suggests that altering dietary protein levels would affect the metabolism and toxicity of heptachlor. The purpose of these studies was to determine the effects of varying dietary casein levels on the enzymatic conversion of heptachlor to its epoxide in weanling rats.

EXPERIMENTAL

Toxicity studies. Male weanling rats, Sprague-Dawley derived,[3] weighing 45 to 55 g were randomly placed in individual stainless steel cages and fed semipurified

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[3] Obtained from Dublin Animal Laboratories, Dublin, Va.

diets for 10 days. Water was provided ad libitum.

The rats were divided into four groups, each consisting of 50 rats, and fed according to the following protocol: three groups were pair-fed and received either 5, 20 or 40% vitamin-free casein. Group 4 received 20% casein ad libitum. The basal diet consisted of the following: (in percent) sucrose, 69.6; vitamin-free casein, 20.0; corn oil, 4.0; minerals,[4] 4.0; vitamins,[5] 2.2; and *dl*-methionine, 0.2. In preparing the other diets, casein and sucrose were varied to provide the desired casein level.

After 10 days on their respective diets, heptachlor[6] was administered by intraperitoneal injection using five levels of the toxicant with 10 animals/treatment level. Heptachlor was administered in a solution of Tween 80 (polyoxyethylene sorbitan monooleate) and saline (20:80) with each rat receiving 0.5 ml/100 g body weight. All animals were held for 96 hours following injection and all deaths occurred within 84 hours. The LD_{50} values were calculated by probit analysis.

A similarly designed experiment was conducted to determine the acute toxicity of heptachlor epoxide. Due to the lower solubility of the epoxide it was administered in Tween 80 and saline (30:70) with each rat receiving 1.0 ml/100 g body weight.

Metabolism studies. Male weanling rats were pair-fed according to the protocol used in the toxicity studies. On the tenth day 10 groups were randomly selected from among the 150 pair-fed animals. Each group consisted of 15 animals, 5 from each diet. Heptachlor was injected intraperitoneally (20 mg/kg) and the rats were decapitated at the following time intervals: zero, 2, 4, 8, 15, 30, 45, 60, 90 and 120 minutes. Blood was collected in oxalated tubes and the livers were immediately excised; they were weighed, minced and frozen until analysis. The liver and blood samples from the five rats fed each diet, at each time interval, were pooled for analysis. Heptachlor and heptachlor epoxide were extracted from blood using the procedure of Jain et al. (9). Residues were extracted from liver by homogenizing the tissue in a glass-Teflon homogenizer with *n*-hexane. After centrifuging the supernatant at approximately 400 × *g* for 10 minutes and evaporating just to dryness, the residue was taken up in 10 ml of *n*-hexane and cleaned up in a co-distillation[7] unit. The eluates were evaporated just to dryness and made to volume for gas-chromatographic analysis. Those samples to which known amounts of heptachlor and heptachlor epoxide were added showed a recovery of 85 to 90% for blood and 72% for liver tissues. Samples were not corrected for incomplete recovery. A gas chromatograph[8] equipped with a ^{63}Ni electron-capture detector and a 6-foot glass column packed with a 3.8% EC-30 on Chromosorb W was used with nitrogen as the carrier gas at a flow rate of 800 ml/minute. The detector was operated at 275° and the column oven at 200°. Portions of the pooled liver sample were analyzed for moisture, Kjeldahl nitrogen and ether-soluble extract (10). Liver residues are expressed as micrograms per gram dry liver protein and blood residues are given per milliliter of blood.

RESULTS

The acute toxicity studies revealed that dietary protein level had a two- to threefold effect on heptachlor toxicity, but not on the toxicity of heptachlor epoxide. The LD_{50} values are shown in table 1.

The differences in the toxicity of heptachlor to the pair-fed rats were reflected in subsequent metabolism studies. Those rats which received 20% casein showed that liver heptachlor increased rapidly for 30 minutes following injection, reaching

[4] Jones-Foster salt mixture, obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The mixture contained: (in percent) sodium chloride, 13.9; potassium phosphate (KH_2PO_4), 38.9; magnesium sulfate, 5.73; calcium carbonate, 38.1; ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 2.70; potassium iodide, 0.079; manganese sulfate ($MnSO_4 \cdot 2H_2O$), 0.445; zinc chloride, 0.025; copper sulfate ($CuSO_4 \cdot 5H_2O$), 0.047; and cobalt chloride, 0.022.

[5] Vitamin diet fortification mixture, obtained from Nutritional Biochemicals Corporation. The mixture contained: (in percent) vitamin A conc (200,000 units/g), 4.50; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.00; ascorbic acid, 45.00; inositol, 5.00; choline chloride, 75.00; riboflavin, 1.00; menadione, 2.25; *p*-aminobenzoic acid, 5.00; niacin, 4.50; pyridoxine·HCl, 1.00; thiamine·HCl, 1.00; and calcium pantothenate, 3.00; and (in milligrams per 100 g diet) biotin, 20.00; folic acid, 90.00; and vitamin B_{12}, 1.35.

[6] Heptachlor and heptachlor epoxide were generously provided by Velsicol Chemical Corporation, Chicago, Ill.

[7] Kontes Sweep Co-Distiller. Manufactured by Kontes, Vineland, N. J.

[8] Micro Tek MT 220, Tracor, Inc., Austin, Tex.

TABLE 1

Comparative LD_{50} values for heptachlor and heptachlor epoxide (mg/kg body wt)

Dietary casein	No. of rats	Heptachlor	No. of rats	Heptachlor epoxide
%				
5 (pair-fed)	50	[1] 97.4 ± 1.8	48	12.7 ± 2.8
20 (pair-fed)	50	30.6 ± 1.7	50	13.0 ± 1.6
40 (pair-fed)	50	28.6 ± 1.9	48	12.8 ± 1.4
20 (ad libitum)	50	74.1 ± 1.4	50	13.6 ± 1.3

[1] LD_{50} ± SE.

a peak of 17.9 μg/g protein (fig. 1). The heptachlor subsequently declined to a level of approximately 5 μg/g protein at 120 minutes. The epoxide appeared at 2 minutes, peaked at 45 minutes (19.4 μg/g protein) and then declined slightly during the subsequent time period.

Rats receiving 5% casein showed an increase of liver heptachlor for 45 minutes when 22.3 μg/g protein was detected (fig. 2). Less conversion to heptachlor epoxide occurred in this group, with the highest residue (10.5 μg/g protein) observed at 60 minutes.

Figure 3 shows that the 40% casein group metabolized heptachlor in a manner similar to the 20% casein group. The highest concentration of heptachlor (18.8 μg/g protein) was detected at 30 minutes and the epoxide peaked between 30 and 60 minutes.

In figure 4 the blood residues for the 5 and 20% casein groups are given. These data show that the appearance of heptachlor epoxide in the blood of the 5% casein group was retarded and did not appear until 60 minutes postinjection. The 20% casein group showed a different pattern with heptachlor peaking at 45 minutes and the metabolite at 90 minutes. Again, the 40% casein group showed responses similar to the 20% casein group.

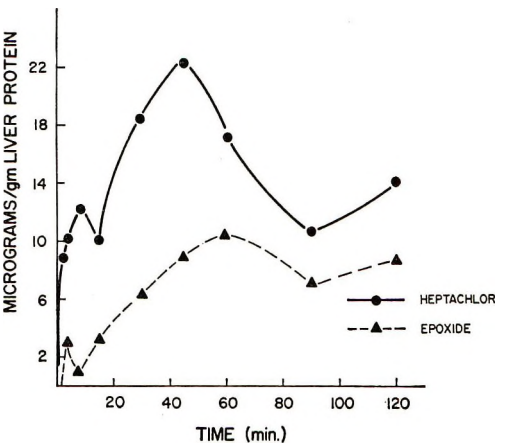

Fig. 2 Effect of 5% dietary casein on the epoxidation of heptachlor in the liver.

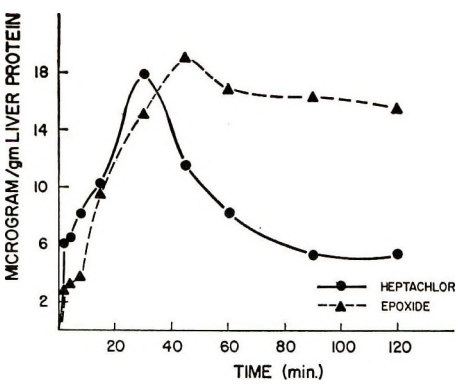

Fig. 1 Effect of 20% dietary casein, pair-fed to rats receiving 5% dietary casein, on the epoxidation of heptachlor in the liver.

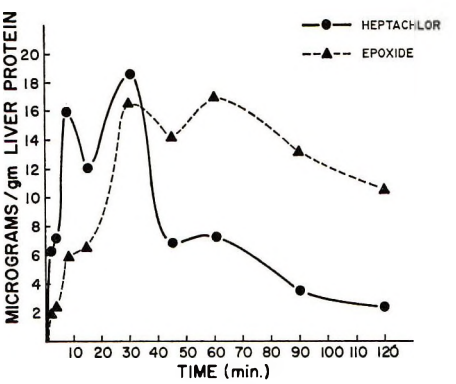

Fig. 3 Effect of 40% dietary casein, pair-fed to rats receiving 5% dietary casein, on the epoxidation of heptachlor in the liver.

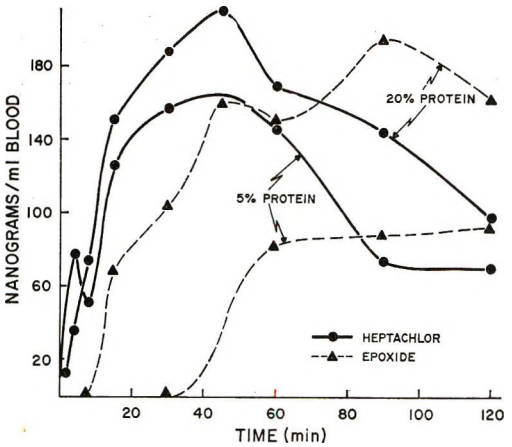

Fig. 4 Effect of dietary casein on the appearance of heptachlor and its epoxide in the blood.

Feeding the three diets resulted in differences in Kjeldahl nitrogen and ether extract fractions whereas the moisture contents showed little or no difference. These data are summarized in table 2.

DISCUSSION

These data indicate that the dietary protein level has a significant effect on the toxicity of heptachlor in the rat. Those rats which were fed 5% casein diets were much less susceptible to heptachlor than were their pair-fed mates receiving the 20 and 40% levels. The rats fed 5% casein also showed a much slower rate of heptachlor metabolism as evidenced by the lower epoxide-to-heptachlor ratios obtained during the time study. Since these rats had only two-thirds as much liver Kjeldahl nitrogen as their pair-fed partners, it is probable that the level of their microsomal oxidative enzymes was also lowered. This would result in a slower conversion to the epoxide, and hence a lower toxicity. Conney (11) stated that microsomal enzymes have very low activity at birth and increase to maximal activity in the adult rat. The weanling rats placed on the 5% casein diet would have two factors retarding the development of microsomal enzymes: 1) a low protein diet, and 2) a low innate activity due to immaturity. Furthermore, the lower level of liver nitrogen in the 5% casein group most likely includes less microsomal enzyme protein, which in turn results in differences in the LD_{50} values for the pair-fed rats Caution should be used when making conclusions of this nature, however, since the toxicity of a substance results from the total response of the animal (12), and the microsomal enzyme level is but a single factor to be considered.

To some extent, another factor responsible for the lower toxicity in the 5% casein group may be the fatty livers characteristic of low protein diets. The highly nonpolar heptachlor may have been trapped in the fat and was therefore not available to the epoxidase system. Data in table 2 indicate that the low protein-fed group had a three- to fourfold increase in ether-soluble extract. Inspection of the data obtained from the metabolism study indicated that the blood from the group fed 20% casein contained more total residue (heptachlor + heptachlor epoxide) than did the 5% group. Possibly, this indicates that some heptachlor was trapped by liver adipose tissue and was not available for circulation. This could mean that the lower rate of epoxidation by the group fed 5% casein results from both lowered microsomal enzymes and greater storage of heptachlor in fat deposits present in the liver.

The difference in toxicity of heptachlor between the group pair-fed 20% casein

TABLE 2

Percentage Kjeldahl nitrogen, ether extract and moisture in livers of rats pair-fed varying levels of casein

Dietary casein	Kjeldahl nitrogen	Ether extract	Moisture
%	%	%	%
5	6.3 ± 0.8 [1]	32.6 ± 4.0	61.2 ± 4.0
20	9.1 ± 0.4	9.1 ± 3.1	66.8 ± 2.4
40	10.3 ± 0.6	7.9 ± 1.9	66.5 ± 1.8

[1] Mean value ± SD. Nitrogen and ether extract values are on a dry weight basis.

and the group fed 20% casein ad libitum is not easily resolved. Both groups received the same protein, but the pair-fed group was subjected to restricted food intake. Dixon et al. (13) and Hospador and Manthei (14) showed that starvation decreased microsomal detoxication of some drugs, but work by Kato (15) and Kato and Takanaka (16) indicated that starvation may induce some microsomal enzyme systems. If starvation increased the activity of the heptachlor epoxidase system, this difference may be explainable.

If, as the above data indicate, the toxicity of heptachlor is dependent on the rate of epoxide formation, the toxicity of heptachlor epoxide should be independent of factors influencing epoxidation. The data derived from the heptachlor epoxide LD_{50} study support this hypothesis. The heptachlor epoxide was more toxic than the parent compound, as expected, and its toxicity was not altered by dietary protein levels. These data confirm the observations of Dahm and Nakatsugawa (17) which indicated that the epoxide was not further metabolized. If the epoxide did undergo further metabolism, the possible induction of hepatic microsomes by starvation should have produced differing toxicities toward the rats which received the two 20% casein diets. The fact that there were no differences in the epoxide toxicities indirectly indicates that the fat deposits in the livers of rats fed low protein do not affect the rate of heptachlor metabolism, as discussed above.

LITERATURE CITED

1. Phillips, W. E. J. 1963 DDT and the metabolism of vitamin A and carotene in the rat. Can. J. Biochem. Physiol., *41:* 1793.
2. Lee, M., K. Harris and H. Trowbridge 1964 Effect of the level of dietary protein on the toxicity of dieldrin for the laboratory rat. J. Nutr., *84:* 136.
3. Krishnamurthy, K., T. S. Subramanya Raj Urs and P. Jayara 1965 Studies on the effect of insecticidal residues in foods. Part I. Effect of poor rice diet on the toxicity of dieldrin to albino rats. Indian J. Exp. Biol., *3:* 168.
4. Stickel, W. H., W. E. Dodge, W. G. Sheldon, J. B. DeWitt and L. F. Stickel 1965 Body condition and response to pesticides in woodcocks. J. Wildlife Manage., *29:* 147.
5. Donaldson, W. E., T. J. Sheets and M. D. Jackson 1968 Starvation effects on DDT residues in check tissues. Poultry Sci., *47:* 237.
6. Boyd, E. M., and E. S. DeCastro 1968 Protein deficient diet and DDT toxicity. Bull. World Health Organ., *38:* 141.
7. Boyd, E. M., and C. P. Chen 1968 Lindane toxicity and protein deficient diets. Arch. Environ. Health, *17:* 156.
8. Wong, D. T., and L. C. Terriere 1965 Epoxidation of aldrin, isodrin, and heptachlor by rat liver microsomes. Biochem. Pharmacol., *14:* 375.
9. Jain, N. C., C. R. Fontan and P. L. Kirk 1965 Simplified gas chromatographic analysis of pesticides from blood. J. Pharm. Pharmacol., *17:* 362.
10. Association of Official Agricultural Chemists 1960 Official Methods of Analysis, ed. 9. Washington, D. C.
11. Conney, A. H. 1967 Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev., *19:* 317.
12. Terriere, L. C. 1968 The oxidation of pesticides: the comparative approach. In: Enzymatic Oxidations of Toxicants. Proceedings of a conference held at North Carolina State University, Raleigh, North Carolina.
13. Dixon, R. L., R. W. Schultice and J. R. Fouts 1960 Factors affecting drug metabolism by liver microsomes. IV. Starvation. Proc. Soc. Exp. Biol. Med., *103:* 333.
14. Hospador, M. A., and R. W. Manthei 1968 Influence of age and diet on the induction of hexobarbital-metabolizing enzymes in the mouse. Proc. Soc. Exp. Biol. Med., *128:* 130.
15. Kato, R. 1967 Effect of phenobarbital treatment on the activities of NADPH-dependent enzymes of liver microsomes in fasted or sucrose fed rats. Jap. J. Pharmacol., *17:* 181.
16. Kato, R., and A. Takanaka 1967 Effect of starvation on the *in vivo* metabolism and effect of drugs in female and male rats. Jap. J. Pharmacol., *17:* 208.
17. Dahm, P. A., and T. Nakatsugawa 1968 Bioactivation of insecticides. In: Enzymatic Oxidations of Toxicants. Proceedings of a conference held at North Carolina State University, Raleigh, North Carolina.

Effect of Coprophagy on Experimental Iron Absorption in the Rat [1]

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ABSTRACT In a 7-day experiment of radioiron absorption (ferrous-59 sulfate) in mature male Fischer rats the effect of coprophagy was studied in three groups: the control group; a group prevented from practicing coprophagy by anal tail cups; and a group with sham tail cups. Hematocrits were similar and normal in all. There was no difference in iron absorption between the "cupped" groups. The greater iron absorption of the control group was of borderline statistical significance.

In a study of absorption of ferrous-59 sulfate following gastric instillation in germfree and conventionalized rats we encountered and discussed a possible effect of coprophagy on absorption (1). This report describes our experience in a similar experiment with radioiron performed on conventional open-animal-room rats in which coprophagy was prevented by an anal cup or cylinder attached to the tail.

MATERIALS AND METHODS

In two replicate experiments 24 mature male rats of the Fischer strain [4] were separated into three groups: control group; a group with coprophagy-preventing anal tail cups which will be described; and a group with sham cups. The average body weight at the time of the experiment was about 300 g, but the animals were deliberately sorted so that the cupped rats were somewhat heavier. It was anticipated that the cupped animals would lose some weight during the 3-week experimental period. The mean body weight at the beginning of the experiment was 268 g for control rats (group A); 300 g for those with sham anal cups during the radioiron absorption experiment (group B); and 305 g for group C which was to be prevented from practicing coprophagy by the anal tail cupping device. All the animals were individually housed on raised no. 2 mesh stainless steel wire flooring and were fed a special formula Purina Laboratory Chow (formula 5010C [5]). Its iron content was 20.2 mg/100 g dry weight. Following receipt of the rats from the supplier, the animals were undisturbed for 1 week to allow them to become adjusted to the diet and local environment. At the beginning of the experiment the animals in groups B and C were "cupped" with a device designed by Barnes et al. in collaboration with investigators from the National Institutes of Health (2). During several years use in our laboratory a few minor changes were made. The cup consisted of a transparent rigid plastic cylinder flared at its open end which spread outward over the anal surface. The edge was polished smooth with emery cloth to prevent irritation of the peri-anal tissue on which it abutted. The distal end was closed by a soft plastic cap perforated in its center to slip over the tail. The cup was kept in place by a safety pin attachment to the raised seam of a rubber sheath cemented to the tail. The perforation in the plastic cap was enlarged in the shape of a keyhole to accommodate the raised seam of the rubber sheath. For 2 weeks prior to gastric instillation of radioiron the animals in groups B and C were cupped with sham tail cups to allow them to become familiar with the device. The sham cup differed from the true tail cup in that one-half of the proximal wall of the bottom was cut out over about two-thirds of its

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[4] Charles River Laboratories, Inc., Wilmington, Mass.

[5] Ralston Purina Company, St. Louis, Mo.

length, and an equivalent amount of plastic tube was attached by a screw to the top of the cup so as to make it the same weight as the true cup. The sham cup permitted easy access of the animal to its anus and feces. All tail cups were inspected twice daily and cleaned at least once daily. At the time of gastric intubation group B was continued on the sham cup regimen to determine any effect on iron absorption of the cupping device unrelated to coprophagy. Group C was switched at this time to the coprophagy-preventing cup.

At the start of the iron-absorption study, after 24 hours without water and food, each animal was given 0.23 μCi ferrous-59 sulfate by gastric instillation. Each dose of radioiron [6] was dissolved in 1 ml distilled water to which 5.0 mg vitamin C and 0.24 mg ferrous sulfate had been added as carrier iron. Within 2 hours postinstillation whole-body counts of radioactive emission were obtained on each animal in a small animal whole-body scintillation counter with attached scaler and timer module.[7] Counts were repeated at the same time daily. The reference standard was counted each time ^{59}Fe retention was measured. All animals were killed by decapitation following light ether anesthesia on day 7 postinstallation, immediately after the final whole-body count was determined.

In four animals of each group, under ether anesthesia and just before decapitation, the abdomen was quickly opened and the duodenum incised 2.0 cm from the pylorus. A glass electrode, 3.5 mm in diameter, was inserted into the lumen and the pH determined at two points on each animal, 1.0 and 4.0 cm distal to the pylorus.[8]

Blood samples were taken on all animals for hematocrit determination at the time of killing. Following decapitation the entire gastrointestinal tract was tied off at the upper and lower ends; it was weighed and its radioactivity determined. Then it was opened, gently washed clean of contents, dried with paper toweling, reweighed and its radioactivity determined in an empty state. The difference between the radioactivity of the filled and empty gastrointestinal tract was subtracted from the whole-body total to obtain the true whole-body absorbed level.

RESULTS

During the 2-week adjustment to the anticoprophagy device by the sham cups the only clinical systemic effect was that body weight gain was less in the two groups on the anticoprophagy device. General appearance, vigor and activity seemed unaffected. The mean weight gain in the controls (group A), was 20.9 g ± 12.9 (SD). In those with anticoprophagy tail cups (group C) the corresponding values were 0.2 g ± 7.4; those with sham cups (group B) had a mean weight gain of 7.6 g ± 12.9. The difference between the controls and those with the coprophagy-preventing cups was statistically significant $P < 0.005$; the other group differences were not significant.

During the week after radioiron administration all animals lost weight, but the mean loss was greater in both groups on the cupping device. In group A (controls) it was 3.6 g ± 4.71; in group B with sham cups it was 6.9 g ± 5.88; and in group C it was 8.9 g ± 10.73.

The means of the body weights at killing were 285 g ± 8.4 for the controls; 296 g ± 16.7 for the group with anal cups; and 300 g ± 15.7 for those with sham cups. The difference was significant between the controls and the group with sham cups: P between 0.025 and 0.05. The difference was not significant between controls and the group with the true anal cups, i.e., P between 0.10 and 0.20.

Figure 1 illustrates the daily whole-body counts less background, but uncorrected for decay in the three groups. In table 1 the data are tabulated for the three groups at the end of the experiment. They include the means of the percentage absorption, after subtraction of gut contents and correction for decay; also the standard deviation and standard error. The values for percentage absorption are higher in all three groups than those reported for open-animal-room rats by others (3). The difference in absorption on the final day between the controls and the other two groups is of borderline significance; i.e.,

[6] Radioiron was obtained from Abbott Laboratories, North Chicago, Ill.
[7] Nuclear-Chicago Corporation, Des Plaines, Ill.
[8] Beckman no. 39042 glass electrode for gastrointestinal pH measurements, Beckman Instruments, Fullerton, Calif.

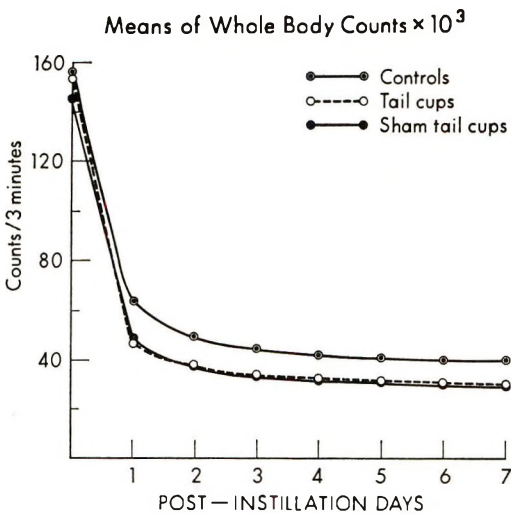

Fig. 1 Effect of coprophagy prevention on ^{59}Fe absorption in rats. Whole-body counts include intestinal contents and are not corrected for decay.

TABLE 1

Percentage absorption of test dose of radioiron [1]

	Group A, Controls [2]	Group B, Sham tail cups	Group C, Tail cups
Mean	29.08	22.48	23.50
SD	6.90	6.13	5.03
SE	2.44	2.17	1.77

[1] From formula: $\frac{\text{Average count for 3 minutes 7 days after instillation (corrected for decay and radioactivity of gastrointestinal contents)}}{\text{Average count for 3 minutes immediately following instillation}} \times 100$

[2] P value between 0.10 and 0.05 for group A versus either group B or group C.

controls versus group with sham cups $t = 2.01$, n = 14 and P between 0.10 and 0.05; controls versus group with coprophagy-preventing tail cups $t = 1.84$, n = 14 and P between 0.10 and 0.05. The difference, however, between the two cupped groups is not significant.

Hematocrits at the time of killing were similar in all three groups: mean of controls, 45.8 ± 2.6%; the group with anal tail cups, 46.4 ± 1.6%; and those with sham cups, mean 45.6 ± 2.0%.

The mean of the duodenal pH determinations in the control group was 7.1 ± 1.0 in the proximal portion and 6.9 ± 0.6 in the distal portion. Corresponding values were 7.3 ± 0.8 and 6.8 ± 0.7 in the group with anal tail cups, and 7.2 ± 0.6 in the proximal duodenum and 7.2 ± 0.5 in the distal duodenum of the group with sham cups.

DISCUSSION

Many experimental nutritionists, notably Elvehjem (4), Daft et al. (5) and Barnes et al. (6) in this country, have shown that intestinal flora may synthesize or destroy nutrients and alter digestive mechanisms. In many animal species a constant recycling of bacteria and their products occurs due to coprophagy. Barnes and associates (6) estimated 50 to 65% consumption of feces by rats and a 15 to 25% decrease in growth rate (2) when coprophagy was prevented by anal cupping.

In a previous experiment with radioiron we observed (1) a sudden abnormally elevated reading during a series of daily whole-body counts in a rat which had had its mesh flooring accidentally dislodged. For only a few minutes the animal had access to fecal material with a high concentration of radioiron in the bottom of the cage. The sudden rise in whole-body count was proved to be due to ingestion of a fecal pellet. Upon immediate killing and necropsy examination the radioactivity of its gastrointestinal contents was found to be elevated to a level corresponding to the abnormal whole-body count.

The results in this experiment, however, show little or no opportunity for testing the effect of coprophagy on iron absorption: there was no difference in whole-body counts between the groups with coprophagy-preventing anal cups and sham cups. If coprophagy had occurred at a 50 to 65% level (6), the daily whole-body counts (fig. 1) should have been higher in the sham cupped group than in those with authentic cups.

Since feeding was resumed after gastric instillation of radioiron, the question has been raised as to the suitability of this experimental model in detecting repassage and absorption of fecal radioiron. It could be argued that isotope dilution by cold iron in the diet might interfere with detection of a difference in absorption even after six passages or cycles.

A difference of borderline statistical significance was demonstrated between controls without cups and the other two groups. This might be attributed to body weight differences since Forrester et al. (3) demonstrated less absorption of radio-iron in larger rats. Another, and perhaps more plausible, explanation would be that it was a nonspecific effect of the cupping devices.

A possible psychologic inhibitory effect of the device on coprophagy in both "cupped" groups must be considered. The experiment was designed to overcome this by a preliminary 2-week adjustment period before starting the iron-absorption study. Also, the design of the sham cup provided easy access to the anus so that fecal pellets could be ingested directly on passing through the anal orifice.

The findings suggest that in short-term experiments such as this, where animals are handled daily and kept on raised wire screen flooring, coprophagy plays no part in iron absorption except as a rare complication.

LITERATURE CITED

1. Geever, E. F., D. Kan and S. M. Levenson 1968 The effect of bacterial flora on iron absorption in the rat. Gastroenterology, 55: 690.
2. Barnes, R. H., G. Fiala and E. Kwong 1963 Decreased growth rate resulting from prevention of coprophagy. Federation Proc., 22: 125.
3. Forrester, R. H., M. E. Conrad and W. H. Crosby 1962 Measurement of total body iron 59 in animals using whole-body liquid scintillation detectors. Proc. Soc. Exp. Biol. Med., *111*: 115.
4. Elvehjem, C. A. 1948 Nutritional significance of the intestinal flora. Federation Proc., 7: 410.
5. Daft, F. S., E. G. McDaniel L. G. Herman, M. K. Romine and J. R. Hegner 1963 Role of coprophagy in utilization of B vitamins synthesized by intestinal bacteria. Federation Proc., 22: 129.
6. Barnes, R. H., G. Fiala, B. McGhee and A. M. Brown 1957 Prevention of coprophagy in the rat. J. Nutr., 63: 489.

Similarity in Passage Rates of Plasma Proteins into the Gut of Germfree and Conventionalized Rats [1,2]

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ABSTRACT Iodine-131-polyvinylpyrrolidone (PVP), ^{131}I-rat albumin and ^{64}Cu-rat ceruloplasmin were injected intravenously in separate experiments into germfree and conventionalized Fischer rats. The decline in whole-body radioactivity and the urinary and fecal excretions of radioactivity were measured. In addition, the half-life of ^{64}Cu radioactivity in the plasma was measured in the rats injected with ceruloplasmin. These experiments were undertaken to determine whether differences in the rate of entry of plasma proteins into the gastrointestinal tract might account in part for the greater fecal nitrogen excretion by germfree rats as compared with their conventionalized or open-animal-room counterparts. The decline in whole-body radioactivity was similar in the germfree and conventionalized rats injected with the ^{131}I-PVP ^{64}Cu-ceruloplasmin, but slightly faster in the conventionalized rats when the ^{131}I-albumin was injected. We found no significant differences in the rate of appearance of radioactivity of the injected tracer compounds in the feces or urine of the germfree as compared with the conventionalized rats. The half-life of ^{64}Cu radioactivity in the plasma was the same in the germfree and conventionalized rats injected with ceruloplasmin. We infer that the rate of entry of plasma proteins into the gut, and the rate of fecal excretion of these compounds and their metabolites, are similar in germfree and conventionalized rats. It is likely, therefore, that these factors do not account for the differences in fecal nitrogen excretion by germfree and conventionalized rats.

A number of years ago, we found that germfree [3] rats excreted more fecal nitrogen than their conventionalized [4] or open-animal-room counterparts (1). This was so when the rats ate isocaloric diets containing 5 to 40% protein, and also during starvation. The experiments reported now were undertaken to determine whether differences in the rates of entry of plasma proteins into the gastrointestinal tract might account in part for the observed differences in fecal nitrogen excretion.

METHODS

Rats of the Fischer strain [5] were housed in flexible vinyl isolators of the type designed by Trexler and Reynolds (2). The husbandry, sterilization and microbiologic techniques used have been described elsewhere (1).

All isotope compounds were made up to a known volume with a 0.85% NaCl solution and pushed through 0.22-μ Millipore [6] membranes into sterile glass ampoules. About 0.8 ml of the isotope solution was injected into the tail vein of a rat which was held in a snug fitting perforated plastic container. The rat in its container was then transferred into a closed tubular extension of the wall of the isolator which could be placed into a whole-body gamma counter [7] for determining the initial whole-body radioactivity.

Daily collections of urine and feces were counted in the same manner along with daily measurements of whole-body radioactivity and a sample of the injected isotopic solution.

When experiments called for the prevention of coprophagy by the rats, plastic tail cups were put on the rats 1 day before the injection of the isotope.

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[3] "Germfree" rats are rats free of viable bacteria, parasites and fungi as judged by methods published elsewhere (8).

[4] "Conventionalized" rats were littermates of the germfree rats contaminated with cecal contents of OAR rats on the day after weaning.

[5] Purchased from the Charles River Breeding Laboratories, Wilmington, Mass.

[6] Millipore Corporation, Bedford, Mass.

[7] Tobor, Nuclear-Chicago, Chicago, Ill.

Rats were housed individually in stainless steel metabolic cages with two-mesh wire floors for 1 day before the injection as well as during the entire experimental period. The rats were fed a steam-autoclaved sterile rat diet (5010C).[8] The ration was coarsely ground and offered at the end of a short tunnel away from the metabolic funnel into which the excreta fell and where the urine was separated from the feces when the rats did not have tail cups (3).

One-half milligram potassium iodide per milliliter drinking water was offered to the rats ad libitum for 72 hours before the injection of ^{131}I-PVP [9] or ^{131}I-rat albumin,[10] and continued during the duration of these experiments to minimize any uptake of iodide by the thyroid. One gram of Rexyn [11] 201 (Cl^-) mixed with 20 g of diet was given to rats daily during the experiment. The resin was sterilized by irradiation by ^{60}Co, 2.5×10^6 rep (93 ergs).

Copper-64-ceruloplasmin was prepared by the method of Morell et al. (4). One hundred thirty-five mCi/20 mg ^{64}Cu [12] were added to 1.0 ml concentrated HNO_3 and heated gently until the copper completely dissolved. The solution was neutralized with NH_4OH to pH 5.0 and brought to a volume of 50 ml with water. About 1.0 ml of the pH 5 solution was injected intraperitoneally into 50 donor rats (about 250 g body weight) at 4 PM. The donor rats ate and drank ad libitum overnight; then they were guillotined at 6 AM the next morning and blood was collected for ^{64}Cu-ceruloplasmin preparation. Two and one-half micrograms of ^{64}Cu-ceruloplasmin in 0.8 ml 0.85% NaCl (about 5.0×10^5 cpm) were injected intravenously into the test rats. Total body counts and fecal and urine counts were done as described above.

RESULTS

Experiments 1 and 2 (table 1). No tail cups were used in experiment 1. The retention of radioactivity in the bodies of both groups of rats during the 4 days following the intravenous injection of ^{131}I-PVP was not significantly different, averaging 26.8% for the germfree and 34.2% for the conventionalized rats. Nor were the average urinary (55.4% for the germfree and 50.8% for the conventionalized) and fecal (17.8% for the germfree and 13.3% for the conventionalized) radioactivity excretions. The ^{131}I accounted for at the end of the study period was 100.0% of the injected dose for the germfree and 98.3% for the conventionalized rats.

Coprophagy-preventing tail cups were applied to all rats in experiment 2 to obviate possible contamination of the fecal specimens by urine during the collections. Fecal radioactivity averaged 5.7% for the germfree and 7.7% for the conventionalized rats for the 4 days following the injection of the ^{131}I-PVP. These values were not statistically different from one another, nor were the urinary excretions (49.7% and 46.9%, respectively) and the whole-body radioactivities (39.9% and 42.8%, respectively). The ^{131}I accounted for at the end of the study period was 95.3% of the injected dose for the germfree and 92.4% for the conventionalized rats.

Experiment 3 (table 2). Iodine-131-albumin was used in this experiment; coprophagy-preventing tail cups were used. There was no significant difference between the germfree and conventionalized rats in regard to fecal excretion of the radioactivity. The fecal radioactivity during the 4 days after injection of the ^{131}I-albumin averaged 3.4% for the germfree and 4.8% for the conventionalized rats. The decline in whole-body radioactivity was moderately faster in the conventionalized rats. An average of 25.4% remained in the body of the germfree at the end of 4 days, and 19.5% in the conventionalized ($P < 0.005$). This was due principally to the slightly greater urinary excretion of radioactivity by the conventionalized rats (73.7% for the germfree and 77.3% for the conventionalized) though this difference was not statistically significant. The ^{131}I accounted for at the end of the study period was 102.5% of the injected dose for the germfree and 101.6% for the conventionalized.

Experiment 4 (tables 3 and 4). Copper-64-ceruloplasmin was used; no tail cups

[8] Ralston Purina Company, St. Louis, Mo.
[9] Radiochemical Centre, Amersham, Buckinghamshire, England.
[10] Rat albumin was bought from Pentex, Inc., Kankakee, Ill. and was iodinated by Abbot Pharmaceuticals at Oak Ridge, Tenn.
[11] Fisher Scientific Company, Fair Lawn, N. J.
[12] Brookhaven National Laboratory, Long Island, N. Y.

TABLE 1

Iodine-131 in gastrointestinal tract of rats [1] following intravenous injection of ^{131}I-PVP [2]

Exp. no.	Days	TBC [3]		P	Urine		P	Feces		P	Total = TBC + urine + feces	
		GF [4]	Conv [4]		GF	Conv		GF	Conv		GF	Conv
		% injected dose			*% injected dose*			*% injected dose*				
1 (no tail cups)	1	47.3 ± 6.1 [5]	50.3 ± 2.7	ns [6]	47.3 ± 4.4	42.7 ± 3.0	ns	3.3 ± 0.8	2.2 ± 0.4	ns	97.9	95.2
	2	35.9 ± 3.8	41.8 ± 0.7	ns	5.1 ± 0.1	4.4 ± 0.2	ns	7.8 ± 6.6	6.8 ± 3.0	ns		
	3	30.8 ± 1.8	36.4 ± 0.3	ns	1.8 ± 0.3	2.2 ± 0.1	ns	4.7 ± 1.5	2.2 ± 0.4	ns		
	4	26.8 ± 1.2	34.2 ± 1.6	ns	1.2 ± 0.1	1.5 ± 0	ns	2.2 ± 0.7	2.1 ± 0.4	ns		
	Total	26.8 ± 1.2	34.2 ± 1.6	ns	55.4 ± 4.2	50.8 ± 3.1	ns	17.8 ± 5.0	13.3 ± 3.6	ns	100.0	98.3
2 (with tail cups)	1	47.5 ± 2.0 [5]	54.6 ± 4.9	ns	45.4 ± 1.2	41.0 ± 1.7	ns	1.3 ± 0.2	1.1 ± 0.2	ns	94.2	96.7
	2	43.6 ± 5.1	48.7 ± 3.0	ns	2.2 ± 0.1	3.0 ± 0.7	ns	1.8 ± 0.3	3.3 ± 0.5	ns		
	3	41.9 ± 1.4	45.4 ± 3.1	ns	1.5 ± 0.3	1.4 ± 0.3	ns	1.5 ± 0.2	1.5 ± 0.3	ns		
	4	39.9 ± 2.0	42.8 ± 3.8	ns	0.7 ± 0.1	1.5 ± 0.4	ns	1.0 ± 0.1	1.8 ± 0.5	ns		
	Total	39.9 ± 2.0	42.8 ± 3.8	ns	49.7 ± 1.2	46.9 ± 2.5	ns	5.7 ± 0.4	7.7 ± 0.8	ns	95.3	92.4

[1] Male rats of the Fischer strain, 8 months old; four in each group.
[2] Isotope obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.
[3] Total body counts.
[4] GF = germfree; Conv = conventionalized.
[5] Mean ± SE.
[6] ns = not significant.

TABLE 2

Iodine-131 in urine, feces and body of germfree and conventionalized rats [1] following intravenous injection of ^{131}I-albumin

Hours	TBC [2]		P	Urine		P	Feces		P	Total = TBC + urine + feces	
	GF [3]	Conv [3]		GF	Conv		GF	Conv		GF	Conv
	% injected dose			*% injected dose*			*% injected dose*				
0–24	66.6 ± 2.5 [4]	61.2 ± 1.1	ns [5]	33.7 ± 1.9	39.4 ± 1.2	ns	0.8 ± 0.2	0.7 ± 0.1	ns	101.0	101.3
24–48	46.8 ± 1.9	44.0 ± 2.1	ns	18.9 ± 0.7	19.0 ± 0.7	ns	1.4 ± 0.2	2.3 ± 0.6	ns		
48–72	33.9 ± 1.5	28.7 ± 0.1	0.02	12.8 ± 0.5	12.5 ± 0.1	ns	0.7 ± 0.1	1.3 ± 0.4	ns		
72–96	25.4 ± 1.4	19.5 ± 0.6	0.005	9.2 ± 1.1	7.9 ± 0.4	ns	0.6 ± 0.1	0.6 ± 0.2	ns		
Cumulative 0–96	25.4 ± 1.4	19.5 ± 0.6	0.005	73.7 ± 2.9	77.3 ± 1.4	ns	3.4 ± 0.2	4.8 ± 0.8	ns	102.5	101.6

[1] Male rats of the Fischer strain, 6 months old; eight in each group.
[2] Total body count.
[3] GF = germfree; Conv = conventionalized.
[4] Mean ± SE.
[5] ns = not significant.

TABLE 3

Copper-64 in urine, feces and body of germfree and conventionalized rats [1] following intravenous injection of ^{64}Cu-ceruloplasmin

Hours	TBC [2]		P	Urine		P	Feces		P	Total = TBC + urine + feces	
	GF [3]	Conv [3]		GF	Conv		GF	Conv		GF	Conv
	% injected dose			*% injected dose*			*% injected dose*				
0–17	94.4 ± 0.7 [4]	94.9 ± 2.2	ns [5]	1.4 ± 0.2	0.4 ± 0.2	ns	1.6 ± 0.6	1.1 ± 0.3	ns	97.4 ±	96.4
17–40	85.2 ± 0.8	85.5 ± 1.1	ns	2.2 ± 0.7	2.3 ± 0.8	ns	6.3 ± 1.5	6.2 ± 1.1	ns		
40–65	79.3 ± 2.1	76.1 ± 2.9	ns	3.0 ± 1.1	2.1 ± 1.2	ns	6.3 ± 1.1	7.1 ± 3.4	ns		
Cumulative 0–65	79.3 ± 2.1	76.1 ± 2.9	ns	6.6 ± 1.1	4.9 ± 1.5	ns	14.2 ± 2.2	18.1 ± 2.6	ns	100.1	99.1

[1] Male rats of the Fischer strain, 9 months old; four in each group.
[2] Total body counts.
[3] GF = germfree; Conv = conventionalized.
[4] Mean ± SE.
[5] ns = not significant.

TABLE 4

Half-life of ^{64}Cu-cerulopasmin in germfree and conventionalized rats [1]

Rat no.	Microbial status	
	Germfree	Conventionalized
	hours	*hours*
1	17.5	19.5
2	—	19.5
3	23.8	18.7
4	18.0	18.0
5	15.5	—
6	16.0	23.5
Mean ± SE	18.2 ± 4.6	19.9 ± 1.0
P	ns	

[1] Female rats of the Fischer strain, 10 weeks old; germfree and conventionalized rats, paired littermates.

were applied. There were no differences between the germfree and conventionalized rats in regard to urinary and fecal excretions of the radioactivity or whole-body radioactivity. The fecal radioactivity excreted in the 4 days following injection of the ^{64}Cu-ceruloplasmin averaged 14.2% of the injected dose for the germfree rats and 18.1% for the conventionalized. In this experiment the half-life of the serum radioactivity was measured. This averaged 18.2 hours in the germfree and 19.9 hours in the conventionalized rats, values which were not significantly different. At the end of the study period, the ^{64}Cu accounted for was 100.1% of the injected dose for the germfree and 99.1% for the conventionalized rats.

DISCUSSION

It is apparent from these studies using three different tracers (^{131}I-PVP, ^{131}I-albumin and ^{64}Cu-ceruloplasmin) that there is no difference in the rate of appearance of the radioactivity of these compounds (and therefore presumably of native plasma proteins) in the feces of germfree and conventionalized rats. The somewhat faster rate of decline in total body radioactivity of the conventionalized rats following the injection of ^{131}I-albumin, as compared with the germfree rats, was as mentioned principally due to their slightly faster urinary excretion of radioactivity. We are inclined to consider the difference in total body radioactivity decline of little importance, particularly since there were no statistically significant differences in the urinary and fecal radioactivity excretions between the two groups.

The limitations and virtues of these compounds for studies of the entry of plasma proteins into the gut have been discussed by a number of investigators (5–8). We had fed ion-exchange resins to the rats in the ^{131}I-albumin and ^{131}I-PVP experiments but we felt that the ^{64}Cu-ceruloplasmin offered an advantage in regard to possible absorption of radioactivity from the gut after the labeled compound had entered the gastrointestinal tract. Also, the ^{64}Cu-ceruloplasmin was endogenously labeled. Another advantage of the ^{64}Cu-ceruloplasmin is that much less of its radioactivity is excreted in the urine than that of the ^{131}I-PVP and ^{131}I-albumin. Thus, the possibility of contamination of the feces by urinary radioactivity when these are collected in the usual metabolic cages is minimized. This possibility of urine-feces cross-contamination is of particular importance in the germfree rats because their feces are stickier than those of the conventionalized and cling more to the sides of the collecting funnel. Such contamination may have occurred in our first experiment with ^{131}I-PVP since the apparent fecal radioactivity was somewhat higher than Sullivan (9) reported for ordinary open-animal-room rats, and than we found in subsequent experiments with ^{131}I-PVP and ^{131}I-albumin when coprophagy-preventing tail cups were used. If this alone were the case, however, one would have expected the urinary radioactivity to be proportionately lower in the cupped rats, but this was not so. It is possible that the difference in fecal radioactivity in the cupped and uncupped rats receiving ^{131}I-PVP was not due to contamination of the feces by urine in the first experiment, but that the wearing of the coprophagy-preventing cups might have induced certain physiologic changes, e.g., a slowing of the entry of the tagged compound into the gut of both the germfree and conventionalized rats. The decline in whole-body radioactivity was somewhat slower in the cupped rats (13% for the germfree, $P < 0.02$; 8.6% for the conventionalized, P not significant) which suggests that there may have been some difference in the way

the rats in the two experiments handled the injected ^{131}I-PVP.

No tail cups were used for the experiment with ^{64}Cu-ceruloplasmin because very little of the label is excreted in the urine.

In all experiments, then, regardless of whether tail cups were or were not used, and regardless of which labeled compound was used, the amount of radioactivity which appeared in the feces following the intravenous injection of the labeled compound was similar in the germfree and conventionalized rats. We conclude that these experiments indicate that the rate of entry of plasma proteins into the gut are similar in the germfree and conventionalized rats. It is likely, therefore, that the entry of plasma proteins into the gut does not account for the differences in fecal nitrogen excretion by germfree and conventionalized rats.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. 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.
2. Trexler, P. C., and L. I. Reynolds 1957 Flexible film apparatus for the rearing and use of germfree animals. Appl. Microbiol., *5:* 406.
3. Barnes, R. H., G. Fiala and E. Kwong 1963 Decreased growth rate resulting from prevention of coprophagy. Federation Proc., *22:* 125.
4. Morell, A. G., P. Aisen and I. H. Scheinberg 1962 Is ceruloplasmin an ascorbic acid oxidase? J. Biol. Chem., *237:* 3455.
5. Gordon, R. S., Jr. 1959 Exudative enteropathy: abnormal permeability of the gastrointestinal tract demonstrable with labelled polyvinylpyrrolidone. Lancet, *1:* 325.
6. Jeejeebhoy, K. N., and N. F. Coghill 1961 The measurement of gastrointestinal protein loss by a new method. Gut, *2:* 123.
7. Waldman, T. A., A. G. Morell, R. D. Wochner, W. Strober and I. Sternlieb 1967 Measurement of gastrointestinal protein loss using ceruloplasmin labelled with copper. J. Clin. Invest., *46:* 10.
8. Jones, J. H., and D. B. Morgan 1963 Measurement of plasma-protein loss into gastrointestinal tract using I-labelled proteins and oral Amberlite resin. Lancet, *1:* 626.
9. Sullivan, M. F. 1960 Polyvinylpyrrolidone labelled with ^{131}I as an agent for the diagnosis of radiation injury in rats. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med., *2:* 393.

Synthesis and Distribution of Cholesterol, and the Effect of Diet, at the Liver Endoplasmic Reticula and Plasma Membranes from Lean or Obese Rats [1,2]

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ABSTRACT To determine the significance of changes in cholesterol concentrations in various membranous components of liver cells, lean as well as genetically obese rats were fed diets containing cholesterol. Cholesterol was found to accumulate to a greater extent in the smooth vesicles (versus rough vesicles) of the endoplasmic reticulum, and the highest concentrations were in the plasma membrane fraction. These increases included a significant proportion of esterified cholesterol. Using ^{14}C-mevalonate in vivo in rats fed a cholesterol-free diet, it was concluded (from the total activities of the isolated labeled cholesterol up to 180 minutes) that in time the total activity of newly synthesized cholesterol shifts from rough vesicles to smooth vesicles and finally to the plasma membrane fraction. However, ^{14}C-cholesterol incorporation in vivo revealed a pattern of incorporation that was similar for smooth and rough vesicles in rats fed diets with or without cholesterol. Total activity of the rough was always higher. From the analytical data, esterification perhaps takes place at the level of the smooth vesicles (or cholesterol ester accumulation hastens the transition of rough to smooth). Polysomal profile analyses indicated that cholesterol feeding resulted in a relative decrease in larger aggregates in obese rats.

Genetically obese rats of the Zucker strain are of interest in studies on lipid metabolism because they tend to have elevated serum and liver lipids (1), as well as higher levels of hepatic protein synthesis and DNA-dependent RNA polymerase activity, than seen in normal lean rats (2, 3). After obese or lean rats have been maintained for short periods of time on diets containing cholesterol, hepatic protein synthesis, RNA polymerase activity and RNA synthesis in vivo are decreased (4). In attempts to explain these phenomena, various possibilities have been considered. One approach is to study changes in the membranous components of the liver cell (5). In the present report, cholesterol feeding procedures have been used to study changes at the endoplasmic reticulum (ER) in both lean and genetically obese rats. This work includes an examination of the cholesterol and protein concentrations of both granular (rough) ER and agranular (smooth) ER, and a study of polysomal profiles. Also, the changes in both total and unesterified cholesterol have been examined in plasma membranes. Finally, the incorporation of ^{14}C into cholesterol has been followed in rough and smooth ER and the plasma membrane fraction, using labeled mevalonic acid or cholesterol.

MATERIALS AND METHODS

Female obese or lean rats of the Zucker strain,[3] as well as commercial rats of the Charles River strain (12 to 14 weeks of age), were housed in a temperature- and humidity-controlled room and fed Purina Laboratory Chow [4] prior to the introduction

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[1] Part of this work was begun during the tenure of Louis Charles Fillios as an Established Investigator of the American Heart Association and was supported by grants-in-aid (HE 11073 and HE 07327) from the Heart Institute of the National Institutes of Health and general research support from University Hospital.

[2] Part of these data were included in a talk given by Louis Charles Fillios at the Conference in Molecular Biology and Pathology at Saratoga Springs, New York on August 2, 1968.

[3] Bred by Dr. Lois Zucker of the Bird Memorial Laboratories, Stow, Mass.

[4] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Mo.

of a purified diet.[5] The purified diet included 20% casein, 20% corn oil, while either cholesterol alone (1.5%) or cholesterol (1.5%) plus cholic acid (0.5%) was added at the expense of carbohydrate level; the detailed composition has been described (4). Rats were fed the purified diets ad libitum for 28 days. Body weight changes during this period remained similar for all dietary groups.

The isolation and analysis of subcellular fractions. After an overnight fast, each rat was killed and the liver was immediately perfused with ice-cold sterile 0.9% saline. The initial procedure used for isolating these liver fractions was modified from that of Hallinan and Munro (6). A fraction of liver (about 4 g) was taken and immersed in ice-cold 0.25 M sucrose and homogenized in cold 0.88 M sucrose using a tissue homogenizer;[6] then the equivalent of 1 g of liver was centrifuged for 30 minutes at 2° at 12,000 × *g* in a centrifuge.[7] The supernatant fraction (18 ml) was then centrifuged at 52,000 × *g* for 90 minutes to obtain rough endoplasmic reticulum. The isolation of smooth endoplasmic reticulum was then carried out by a modification of the method of Dallner et al. (7). The proteins, isolated by precipitation with perchloric acid, were dissolved in 0.3 KOH and aliquots saved for the determination of total protein by the method of Lowry et al. (8). Another 1-g sample of liver was used to isolate the lipids from these subcellular fractions. Following the above procedures, the lipids of the isolated rough and smooth endoplasmic reticulum were extracted with methanol–chloroform (2:1). The residual protein was determined as described above. The lipid extracts were washed twice with 0.2 volume of 0.05% $CaCl_2$ to remove any nonlipid contamination (9). The lipid phase was then collected and evaporated overnight; the dried lipid materials containing cholesterol were reextracted with an acetone–alcohol solution (1:1) according to the method of Shapiro and Kritchevsky (10). After filtration, the acetone–alcohol extracts were collected and portions were taken for further purification and isolation of the cholesterol by digitonide precipitation according to the method of Sperry and Webb (11). Finally, the cholesterol precipitate was dissolved with methanol–alcohol (1:5) and an aliquot saved for the determination of cholesterol (12).

Total rat livers were used for the isolation of the plasma membrane fractions. The isolation was carried out according to a modification of the procedure described by Emmelot et al. (13), which is based on the original method of Neville (14). Again, isolation of cholesterol was carried out after purification and digitonide precipitation; the esterified cholesterol was determined by the difference of the total and free cholesterol; protein determinations, as above.

Electron microscopy. Selected rat livers from lean and obese rats were removed, perfused and sectioned. Small portions were then fixed in 1% osmic tetraoxide, buffered with Millonig's buffer, dehydrated with graded ethanol (50 to 100%), and embedded in Araldite. Thin sectioning was carried out in a Potter-Blum MT-2 microtome with glass knives. Specimens were finally stained with uranyl acetate and lead acetate. Viewing and photography were carried out using an electron microscope.[8]

The electron microscopy was also used to monitor subcellular fractions. The electron micrographs showed that the above isolation procedures gave satisfactory results.

Polysomal profile analysis. From portions of liver (control obese as well as obese fed the test diets for 28 days) polysomes, oligosomes and monosomes were obtained after their isolation by a modification of the sucrose gradient techniques described by Drysdale and Munro (15). This technique first removes interfering ferritin by use of rabbit antiserum. After the deoxycholate-treated fraction was centrifuged, the pellet was gently suspended in a buffered solution and the suspension was layered over a linear sucrose density gradient (10 to 40%) and centrifuged for 1 hour at 110,000 × *g* at 1°, using a swinging bucket rotor. The ultraviolet absorption was measured at 260 mμ after the materials were collected by puncturing the

[5] All dietary ingredients were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.
[6] Tri-R Instruments, Rockville Centre, N. Y.
[7] B-60 Ultracentrifuge, International Equipment Company, Needham Heights, Mass.
[8] Model EMU-2E, Radio Corporation of America, Camden, N. J.

bottom of the tube. A recording spectrophotometer attached to a flow-through cell system was used.

Studies on cholesterol metabolism using 2-^{14}C-mevalonate or cholesterol-4-^{14}C. Each rat was fasted 16 to 20 hours (20 hours at time of killing) and then injected with 2-^{14}C mevalonate.[9] Two minutes after the injection (2 μCi/100 g body weight) of the labeled mevalonate, converted to the menvalonate potassium salt, a 25-fold injection of unlabeled mevalonate salt [10] was given to dilute the labeled pool. Animals were killed at various time intervals up to 3 hours after receiving the labeled material. Serum total cholesterol levels were routinely determined and are in agreement with previous observations for either lean or obese rats (2, 3). For the measuring of the specific activity in the circulating cholesterol, the lipids were extracted and finally the cholesterol was isolated as the digitonide, dried and then redissolved in methanol–alcohol (1:5). An aliquot was taken for cholesterol determinations and another was dried and measured for radioactivity by means of a thin window gas-flow counter.[11] Specific and total activities (based on cholesterol-to-protein ratios) for all subcellular fractions described in this study were calculated.

In a related experiment, each rat received an injection of cholesterol-4-^{14}C (5 μCi/100 g) [12] and was killed at various intervals up to 3 hours. The incorporation of labeled cholesterol expressed as specific activity or total activity was determined for both the smooth and rough vesicles.

Further details are given in the legends of figures 1 and 3 for this phase of the study.

DISCUSSION OF RESULTS

The endoplasmic reticulum (ER) is a major site for protein synthesis, as well as cholesterol synthesis (16–17). The changes that take place at this site have a direct bearing on the events that control the elaboration and secretion of the plasma lipoproteins. A systematic examination of the biochemical changes associated with these organelles, and the more peripheral organization of the liver cell, namely the plasma membrane, is therefore of direct interest in such studies.

Rough vesicles of the ER contain approximately 70% of the total protein associated with the endoplasmic reticulum (6, 18). This distribution remains the same after cholesterol feeding (table 1). The data also reveal that the absolute amount of total protein of the endoplasmic reticulum in the liver is actually increased following cholesterol feeding in the lean rats, but the opposite appears to be the case for obese rats. Despite these differences in response, the relative distribution of protein between these two components of the ER remains approximately the same. The significance of this difference between lean and obese has not been determined, but may be explainable by differences in the turnover rates of membranous protein, or the rate of secretion of plasma proteins between the two genotypes.

The data in table 1 also show an increase in the cholesterol-to-protein ratio for both fractions of the endoplasmic reticulum after cholesterol feeding. The smooth vesicles appear to have consistently higher ratios before and after cholesterol feeding and this agrees with observations on commercial rats [13] from this laboratory (5). Of perhaps more significance, the cholesterol of the rough vesicles is almost entirely in the free form (98%); values for smooth vesicles are somewhat less (91%) (table 2) and of course the circulating cholesterol is predominately esterified. Normally, almost all of the cholesterol content of the liver is in the free form. Following cholesterol feeding, the free cholesterol content increases only moderately, but the excess cholesterol that accumulates is almost entirely esterified. The evidence appears to indicate that the site in the liver for cholesterol esterification is microsomal (19).

[9] 2-^{14}C-Mevalonic acid (specific activity: 5 μCi/μmole) was purchased as the lactone from Calbiochem., Los Angeles, Calif. It was converted to the potassium salt by the method of Shapiro and Kritchevsky (10). Each rat received 2 μmoles of labeled mevalonate in 0.9% NaCl.

[10] Mevalonic acid was also purchased from Calbiochem. After treatment with KOH, the dibenzylethenedione was extracted with petroleum ether, and the mevalonic acid solution was neutralized with HCl. Each rat received 50 μmoles of the unlabeled mevalonate in 0.9% NaCl.

[11] Nuclear-Chicago, Des Plaines, Ill.

[12] Cholesterol-4-^{14}C (specific activity: 50 μCi/μmole) was purchased from New England Nuclear Corporation, Boston, Mass.

TABLE 1

Relationship of cholesterol to protein in the endoplasmic reticulum [1]

Genotype	Dietary treatment	Liver wt	Rough vesicles			Smooth vesicles		
			Cholesterol	Protein	$\frac{\text{Cholesterol}}{\text{Protein}} \times 100$ [2]	Cholesterol	Protein	$\frac{\text{Cholesterol}}{\text{Protein}} \times 100$ [2]
		g	*mg/g liver*	*mg/g liver*		*mg/g liver*	*mg/g liver*	
Fa Fa	Controls	5.7	0.79	20.1	4.1 ± 0.5	0.33	6.8	4.9 ± 0.7
(lean)	Cholesterol + [3]	9.0 [4]	0.97	15.8	6.1 ± 0.4	0.56	5.6	9.9 ± 0.9
fa fa	Controls	17.1	0.73	15.7	4.6 ± 0.4	0.34	5.7	6.0 ± 0.6
(obese)	Cholesterol + [3]	23.8	0.57	6.2	9.0 ± 0.8	0.33	2.7	12.9 ± 1.2

[1] Based on a total of 42 observations; 14 for each control group and 7 for each cholesterol group.
[2] ± SE of the mean.
[3] Cholesterol + represents those rats fed the purified diet containing cholesterol and cholic acid for 28 days.
[4] Underlined values are significantly different from their respective controls ($P < 0.01$).

During cholesterol feeding, the rough vesicles appear to accumulate a significant amount of cholesterol (expressed in terms of the total protein of the polysome-free material), but the smooth vesicles which normally do not have adhering polysomes consistently show an even greater capacity for cholesterol accumulation. This generally agrees with an earlier observation for microsomal total cholesterol levels after cholesterol feeding (20). Data in table 2 indicate that a significant portion of the additional cholesterol, which presumably accumulates within the smooth vesicles, is esterified; in the rough vesicles the cholesterol content remains largely in the free form (over 90% of the total). Normally, the obese rat has been found to have a slightly lower percentage of free cholesterol in the rough vesicles than seen in lean rats, but the differences were not found to be statistically significant. The smooth vesicles of the obese rat, however, have a lower percentage of free cholesterol (86%), suggesting that the esterification process in these animals may be very efficient since they spontaneously also show hypercholesteremia.

To what extent cholesterol is an integral part of the membranous component of the ER is not known. It can be surmised that free cholesterol may normally be an integral part of the membrane, whereas the esterified cholesterol represents a "storage" form. It is also of interest that the amount of free cholesterol normally associated wtih the endoplasmic reticulum of the normal rat represents a significant percentage of total liver cholesterol. The remaining free cholesterol is presumed to be associated with other membranous components in the cell, including the plasma membrane which is relatively rich in cholesterol (13). It is apparent from tables 1 and 3 that there is relatively more cholesterol in the plasma membranes in agreement with such earlier reports, but that this cholesterol is relatively more esterified, as seen in table 2; following cholesterol feeding, the percentage of esterified cholesterol increases further. Therefore, as one moves "peripherally" (from rough ER to

[13] Purchased from Charles River Laboratories, Inc., Wilmington, Mass.; derived from Sprague-Dawley strain.

TABLE 2

Free cholesterol concentrations in membranous fractions (as percentage of total cholesterol)

Genotype	Dietary group	Rough vesicles	Smooth vesicles	Plasma membrane
Fa Fa (lean)	Controls (6) [1]	98 ± 1.1 [2]	91 ± 2.0	87 ± 3.4
	Cholesterol [3] (6)	[4] 91 ± 1.2	78 ± 2.1	70 ± 2.1
fa fa (obese)	Control (6)	94 ± 1.4	86 ± 2.2	81 ± 2.8
	Cholesterol (6)	80 ± 2.3	72 ± 3.0	66 ± 3.0

[1] Values in parentheses represent number of rat livers.
[2] ± SE of the mean.
[3] Cholesterol refers to a purified diet containing 1.5% cholesterol.
[4] Underlined values are significantly different from their respective controls ($P < 0.01$).

TABLE 3

Cholesterol-to-protein ratios in plasma membrane fractions

Genotype	Dietary treatment	$\frac{\text{Cholesterol}}{\text{Protein}} \times 100$
Fa Fa (lean)	Controls (5) [1]	20.2 ± 1.2 [2]
	Cholesterol [3] (5)	[4] 26.7 ± 1.4
	Cholesterol + (4)	31.4 ± 2.4
fa fa (obese)	Controls (5)	18.9 ± 1.1
	Cholesterol (5)	24.7 ± 1.7
	Cholesterol + (3)	30.0 ± 2.2

[1] Number in parentheses represents number of rat livers.
[2] ± SE of the mean.
[3] Cholesterol represents rats fed 1.5% cholesterol; cholesterol + represents rats fed cholesterol plus 0.5% cholic acid.
[4] Underlined values are significantly different from their respective controls ($P < 0.01$).

smooth ER to plasma membrane), the percentage of esterified cholesterol increases, especially after cholesterol feeding. Furthermore, the isotope data, in figure 1, emphasize in a more dynamic way the relative shifts in total activity from rough to smooth to plasma membrane in rats fed control diets. This latter circumstance is supported by a recent report by Chesterton (19) which indicates that the rough, and probably also the smooth, endoplasmic reticulum is the major site for both the synthesis and subsequent esterification of cholesterol. Our electron micrographs show that genetically obese rats have an apparently richer network of rough reticulum in their parenchymal cells with more closely packed parallel cisternae than lean rats.[14] This observation may account for the relatively higher level of protein synthesis and output of plasma proteins seen in this genotype. Following cholesterol feeding, the level of protein synthesis is markedly reduced in such rats (2–4); this decreased activity is reflected by the changes in the polysome profile in figure 2, since optional protein synthesis is presumably dependent on polysomal aggregations (21). An examination of the liver polysome profiles

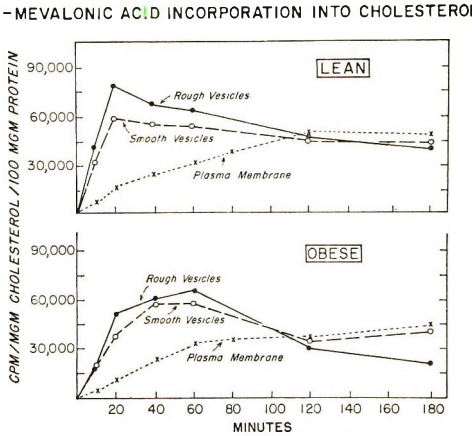

Fig. 1 ^{14}C incorporation into cholesterol digitonide, using 2-^{14}C-mevalonate, to study the total activity of newly synthesized cholesterol in various subcellular fractions.* Each rat received the labeled mevalonate and, 2 minutes later, a diluting dose of unlabeled mevalonate. Two to four rats were killed at the time intervals indicated (each point represents four to eight determinations); a total of 34 lean and 36 obese homozygotes of the Zucker strain (fed the control diet) were required. None of these rats was fed cholesterol. Specific activities alone can be derived from the cholesterol-to-protein ratios in tables 1 and 3.

* The biological half-life of the newly synthesized cholesterol for rough vesicles can be determined from the slope of the lines (on a semi-log plot). Lean rats showed a turnover time of less than 3 hours whereas obese rats had an apparently faster turnover time (less than 2 hours). The effects of cholesterol feeding on the total activity in rough and smooth vesicles following ^{14}C-mevalonate have been described in a Sprague-Dawley strain of rats (5).

[14] Satoh, T., Ira Gore and L. C. Fillios 1968 unpublished data.

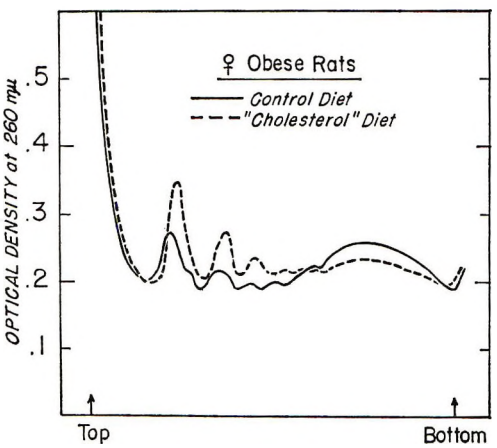

Fig. 2 Polysomal patterns from livers of rats fed the contol diet or a diet containing cholesterol. The postmitochondrial fraction was layered over a continuous sucrose gradient (10 to 40%) after the suspensions were equilibrated in terms of optical density. The right hand of the pattern represents polysomal material; the left hand peak, monosomes. "Cholesterol" refers to a diet containing 1.5% cholesterol.

of obese rats fed the cholesterol-supplemented diet shows a relative decrease of larger aggregates (polysomes) with a concomitant increase in monosomes and possibly disomes and trisomes. Similar observations have also been made in commercial rats (5). Presumably, all or most of the monosomes are cytoplasmic. In support of this, Kim et al. (22) found that the normal endoplasmic reticulum of the rat liver becomes highly disorganized, with the cisternae irregularly distended and many of the ribosomes apparently dispersed into the cytoplasms, following the feeding of a thrombogenic diet containing cholesterol, cholic acid and thiouracil. Although their dietary procedures are quite severe and result in anorexia, somewhat similar observations have been indicated in our observations where dietary treatments are relatively mild. From such observations, one can argue that the accumulation of cholesterol and triglycerides results in a distension of the cisternae and this interferes with the normal protein synthesizing apparatus at the ER, resulting in a disaggregation of the polysomes at the surface. Apparently exogenous or preformed (free) cholesterol (using ^{14}C-cholesterol) accumulates preferentially in the rough ER (fig. 3). Thus, the accumulation and esterification of cholesterol and other lipids may hasten the transition of rough to smooth ER.

It can be visualized that the original lipoprotein membranes from the ER may also serve as vehicles for vesiculation in order to participate in the sequence of "transport" of excess lipids to the periphery of the cell, and their subsequent extrusion into the circulation. Such a hypothesis had been directly or indirectly suggested (2, 5, 23, 24). One can assume that normally, lipoprotein membranes of the smooth ER eventually may be utilized for the "packaging" of esterified cholesterol and other

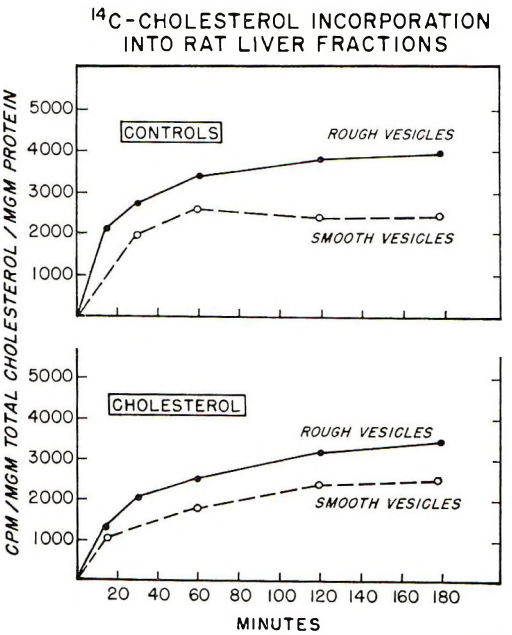

Fig. 3 ^{14}C-cholesterol incorporation into rat liver endoplasmic reticulum. Each point represents four to eight values from two to four rat livers. A total of 30 rats of the Charles River strain were fed either the control or purified diet containing 1.5% cholesterol for 28 days. Apparently the exogenous cholesterol is preferentially incorporated into the rough vesicles. The above data were corrected for cholesterol accumulation per milligram of vesicle protein and thus the total activity for both dietary groups is somewhat similar. "Cholesterol" refers to rats fed cholesterol. Specific activities can be derived from the cholesterol-to-protein ratios in table 1 but would be misleading since these ratios differ between these two vesicle fractions.

lipids. After undergoing modification, these lipoprotein complexes may be extruded in whole or in part into the circulation via the plasma membrane to elaborate the observed spectrum of circulating lipoproteins, the sizes and densities of the lipoproteins being dependent on their random lipid loads. Such a hypothesis is consistent with our present knowledge. Furthermore, it has not been determined exactly how and where the various lipoprotein moieties are assembled prior to their release. Electron microscopy, however, has detected osmophilic bodies (purportedly lipoproteins) associated with smooth elements within the cell and in spaces of Disse (23, 24).

In pursuing this idea, the "chasing" of various labeled components is being studied using the ER as one of the possible intracellular sites that may be involved in this mechanism. The smooth elements, or the Golgi apparatus, and other intracellular membranes, particularly the plasma membrane, should also be considered in this approach. In the present study, labeled mevalonic acid was used to study the postulated "movement" of cholesterol. The accumulation of newly synthesized cholesterol in rough ER appears to take place early. In time, the total activity in the smooth ER equals or exceeds that of the rough ER. The precise route can only be surmised and certainly appears to involve other smooth elements of the cell, such as the plasma membrane. Therefore, the alterations or modifications of the normal liver protein synthesizing apparatus during cholesterosis is closely associated with membranous changes. As lipids accumulate, the synthesis (or activity) of certain proteins (or enzymes, or both), is apparently depressed in favor of these chemical events required for the orderly extrusion of lipids, including cholesterol and cholesterol esters, into the circulation. To what extent and how specific intracellular membranes participate in these events is emphasized in the present study, which shows an apparent sequential movement of cholesterol with esterification taking place presumably at the smooth vesicle level; and finally, the accumulation of free and esterified cholesterol at the plasma membrane prior to its extrusion as a glyco-lipoprotein complex into the circulation.

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LITERATURE CITED

1. Zucker, L. M. 1965 Hereditary obesity in the rat associated with hyperlipemia. Ann. N. Y. Acad. Sci., *131:* 447.
2. Fillios, L. C., and S. Saito 1965 Hepatic protein synthesis and lipid metabolism in genetically obese rats. Metabolism, *14:* 734.
3. Fillios, L. C., and O. Yokono 1966 Liver RNA polymerase activity in genetically obese and lean rats fed cholesterol. Metabolism, *15:* 279.
4. Fillios, L. C., and O. Yokono 1968 Hepatic RNA metabolism in male and female obese rats fed cholesterol. J. Nutr., *95:* 349.
5. Pronćzuk, A., and L. C. Fillios 1968 Changes in cholesterol concentrations in rough and smooth endoplasmic reticulum and polysomal profiles in rats fed cholesterol. J. Nutr., *96:* 46.
6. Hallinan, T., and H. N. Munro 1965 A rapid method for preparing granular and agranular endoplasmic reticulum from rat liver. Quart. J. Exp. Physiol., *50:* 93.
7. Dallner, G., P. Siekievitz and G. E. Palade 1966 Biogenesis of endoplasmic reticulum membranes. J. Cell Biol., *30:* 98.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurements with Folin phenol reagent. J. Biol. Chem., *193:* 265.
9. Folch, J., and M. Lee 1957 A simple method for the isolation and purification of total lipid from animal tissue. J. Biol. Chem., *226:* 597.
10. Shapiro, J. L., and D. Kritchevsky 1963 Radioassay of cholesterol ^{14}C-digitonide. Anal. Biochem., *5:* 88.
11. Sperry, W. M., and M. Webb 1950 Revision of the Shoenheimer-Sperry method for cholesterol determination. J. Biol. Chem., *187:* 97.
12. Bowman, R. E., and R. C. Wolf 1962 Rapid and specific ultramicro method for total cholesterol. Clin. Chem., *8:* 302.
13. Emmelot, P., C. J. Bos, E. L. Benedetti and P. H. Rumke 1964 Studies on plasma membranes. Biochim. Biophys. Acta, *90:* 126.
14. Neville, D. M. 1960 The isolation of a cell membrane fraction from rat liver. J. Biophys. Biochem. Cytol., *8:* 413.
15. Drysdale, J. W., and H. N. Munro 1967 Polysome profile obtained from mammalian

tissue by an improved procedure. Biochim. Biophys. Acta, *138*: 616.
16. Siekievitz, P. 1963 Protoplasm: Endoplasmic reticulum and microsomes and their properties. Ann. Rev. Physiol., *25*: 15.
17. Bucher, N. L., and K. McGarrahan 1956 The biosynthesis of cholesterol from acetate 1-^{14}C by cellular fractions of rat liver. J. Biol. Chem., *222*: 1.
18. Hallinan, T., and H. N. Munro 1964 Comparison of amino acid incorporation and ribonucleic acid turnover in rat liver submicrosomal fraction. Biochim. Biophys. Acta, *80*: 166.
19. Chesterton, C. J. 1968 Distribution of cholesterol precursors and other lipids among rat liver intracellular structures. J. Biol. Chem., *243*: 1147.
20. Scholtz, M. C., L. I. Rice and R. Alfin-Slater 1953 Further studies on cholesterol in liver cell fraction of normal and cholesterol-fed rats. J. Biol. Chem., *204*: 19.
21. Wettstein, F. O., T. Staehelin and H. Noll 1963 Ribosomal aggregates engaged in protein synthesis: Characterization of the ergosome. Nature, *197*: 430.
22. Kim, D. N., K. T. Lee, R. Jones and W. A. Thomas 1966 Studies of fibrinogen synthesis in rats fed thrombogenic diets. Exp. Mol. Pathol., *5*: 61.
23. Jones, A. L., N. B. Ruderman and M. G. Herrara 1966 An electron microscopic study of lipoprotein production and release by the isolated perfused rat liver. Proc. Soc. Exp. Biol. Med., *123*: 4.
24. Hamilton, R. L., D. M. Regen, M. E. Gray and V. S. LeQuire 1967 Electron microscopic identification of very low density lipoproteins in perfused rat liver. Lab. Invest., *16*: 305.

Glycine Metabolism in Vitamin B_6-deficient and Deoxypyridoxine-treated Rats [1]

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ABSTRACT Further studies in support of previous work indicating that vitamin B_6 deficiency and deoxypyridoxine alter the metabolism of glycine by rats have been conducted. Vitamin B_6 deficiency resulted in significant elevation in the free glycine of rat liver, kidney, blood and muscle. This effect was not accentuated by deoxypyridoxine and was prevented in liver and kidney. When vitamin B_6-deficient rats were injected with glycine-1-^{14}C less $^{14}CO_2$ was expired than by controls. Deoxypyridoxine neither reversed nor increased this effect. Rats receiving deoxyridoxine converted less labeled glycine, glyoxylate and glycolate to hippuric acid than vitamin B_6-deficient rats not receiving the analogue. Liver and kidney homogenates from vitamin B_6-deficient rats showed a decreased ability to oxidize glycine-1-^{14}C to $^{14}CO_2$. This did not occur in liver homogenates from rats fed deoxypyridoxine, although kidney homogenates from these rats acted similarly to those from vitamin B_6-deficient animals not receiving deoxypyridoxine. The addition of pyridoxal-5-phosphate to liver homogenates increased glycine oxidation in the homogenates from control rats and from both experimental groups to levels which were not significantly different. In contrast, pyridoxal-5-phosphate did not alter the glycine oxidation of control kidney homogenates and did not raise the activity of kidney homogenates from the deficient groups to control levels.

Prior studies have indicated that vitamin B_6 deficiency in rats (1) is accompanied by oxaluria, and this effect can be enhanced by dietary supplementation with glycine, which acts as an oxalate precursor, or with the vitamin B_6 antagonist, deoxypyridoxine. The effects of deoxypyridoxine in depressing growth and antibody formation and in increasing mortality in vitamin B_6-deficient rats can be partially reversed by feeding glycine or serine (1, 2). Also, the growth-depressing effects of feeding high levels of glycine to vitamin B_6-deficient rats can be reversed by deoxypyridoxine administration. Obviously the effects of deoxypyridoxine are not identical to those of vitamin B_6 deficiency. These studies suggest that in the vitamin B_6-deficient rat receiving deoxypyridoxine a dietary source of glycine or serine, or both, becomes necessary.

Other studies have indicated that alterations of glycine and serine metabolism occur in vitamin B_6-deficient rats. These have included decreased tissue serine levels and increased tissue glycine levels (3), impaired conversion of glycine to CO_2 by liver and kidney homogenates (4), decreased synthesis of glycine from glyoxylic and glycolic acids (4) and decreased incorporation of carbon 3 of serine into choline and creatine (5).

The present work was done to further study the effects of vitamin B_6 deficiency and deoxypyridoxine on the metabolism of glycine and related metabolites.

METHODS

In these studies male weanling rats of the Charles River CD strain were used. They were fed semipurified diets ad libitum containing: (in percent) casein, 25; sucrose, 60.7; corn oil, 9; salts IV (6), 4; cod liver oil, 1; and choline, 0.3. Vitamins were added so that each 100 g of diet contained: (in milligrams) thiamine·HCl, 0.4; riboflavin, 0.8; niacin, 4; calcium pantothenate, 2; folic acid, 0.1; biotin, 0.02; menadione, 0.1; *dl*-α-tocopherol, 10; and vitamin B_{12}, 0.005. Control diets contained 0.4 mg pyridoxine·HCl per 100 g and,

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when used, 12.5 mg of 4-deoxypyridoxine were added per 100 g of vitamin B_6-deficient diet.

In vivo experiments. Hippuric acid: Radioactive compounds were obtained with the following specific activities (microcuries per milligram) and used without further purification for in vivo experiments: glycine-1-^{14}C(68), calcium glycolate-1-^{14}C(76) and sodium glyoxylate-1-^{14}C(42). The labeled compounds injected were dissolved in 0.05 M sodium benzoate for hippuric acid experiments. Each milliliter of solution contained 1 to 7 μCi of radioactivity. The rats were divided into groups of three and housed in metabolism cages so that 24-hour urine collections could be made following intraperitoneal injections of these solutions. Each animal was injected with 0.5 ml of solution per 100 g of body weight. A minimum of 30 hours elapsed between injections when rats were injected with more than one labeled compound. All injections were made when the rats had been fed the experimental diets not less than 5 or more than 9 days. Samples were counted in a scintillation counter [3] using a scintillation solution consisting of 3 ml ethanol plus 6 ml toluene containing 0.4% 2,5-diphenyloxazole and 0.005% *p*-bis-2′-(5′-phenyloxazolyl) benzene; corrections for efficiency were made by the channels ratio method.

Hippuric acid was isolated by adding 2 ml of carrier hippuric acid solution (100 mg/ml in 2 N NaOH) to an aliquot of urine in a 40-ml centrifuge tube. The solution was acidified with 1 ml 5 N H_2SO_4 and was allowed to stand overnight in the cold; it was then centrifuged and the supernatant fraction was discarded. The crystals were dissolved in 5 ml H_2O in a hot-water bath, and the hot solution filtered into a 12-ml centrifuge tube. The samples were allowed to recrystallize overnight in the cold; they were centrifuged and the supernatant solution was discarded. Recrystallization was repeated, omitting the filtering step. The crystals were dried in an oven, weighed, and counted using the same volume and composition of scintillation fluid described above. One drop of 20% NaOH was added to each sample to prevent acid quenching.

Respiratory $^{14}CO_2$: Glycine-1-^{14}C (68 μCi/mg) was dissolved in 0.15 M NaCl solution. Each rat was injected with 0.5 ml solution/100 g body weight. Each milliliter of solution contained 3 μCi of radioactivity. Respiratory $^{14}CO_2$ was collected for 1 hour in NaOH solution and 0.2-ml aliquots of the NaOH solution were counted in counting solution of the same volume and composition as described above.

In vitro experiments. Rats that had been fed the experimental diets for 5 to 7 days were used. They were decapitated; a portion of liver and both kidneys were removed and weighed, and each tissue homogenized in 10 volumes of cold 0.15 M KCl solution. The time between decapitation and the beginning of the incubation was 6 minutes for liver homogenates and 8 minutes for kidney.

Each incubation mixture consisted of 1.0 ml of homogenate plus 2.0 ml of 0.0075 M glycine-1-^{14}C in a buffer prepared by mixing 30 parts of 0.10 M potassium phosphate buffer, pH 7.4, and 70 parts of 0.15 M KCl. The samples were incubated for 1 hour at 37°C in a Dubnoff metabolic shaker set at 100 reciprocations/minute, using 10-ml Erlenmeyer flasks with center wells containing 0.1 ml of 20% NaOH and a filter paper fan to absorb $^{14}CO_2$. The absorbed $^{14}CO_2$ was counted by the method of Buhler (7). The protein content of the homogenates was determined by the method of Lowry et al. (8).

The glycine used in the in vitro experiment was purified as follows: Several milligrams of labeled glycine were mixed with 1 g of unlabeled glycine; the mixture was dissolved in water and recrystallization was induced by the addition of ethanol. The crystals were separated and additional radioactive glycine, recovered from the supernatant solution by addition of 500 mg unlabeled glycine, dissolved in a minimum of water. This recovery step was repeated; the three batches of crystals thus obtained were combined and the entire procedure was repeated.

Tissue glycine. Free tissue glycine levels were determined by the method of Alexander et al. (9) using blood collected from the neck, and homogenates of liver, kidney and skeletal muscle from the hind

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limbs. Tissues were homogenized in a solution of 1% sodium tungstate solution —0.66 N H_2SO_4 (9:1) to obtain a protein-free filtrate.

RESULTS

In vivo experiments. Table 1 indicates that the glycine content of tissues from vitamin B_6-deficient rats was significantly higher than that of tissues from control animals. The time of the appearance of these differences in glycine content, however, varied from tissue to tissue, being seen earliest in muscle and last in liver. Table 2 shows that the addition of deoxypyridoxine to vitamin B_6-deficient diets did not enhance the effect of vitamin B_6 deficiency in increasing tissue glycine levels. Instead it appeared to reverse it. In this study the kidneys and livers were more sensitive to the effect of the deoxypyridoxine than muscle.

Table 3 shows that significantly less labeled hippuric acid was excreted by vitamin B_6-deficient rats than by controls when they were injected with labeled glycolate-1-^{14}C or glyoxylate-1-^{14}C. With each of these compounds the feeding of deoxypyridoxine to vitamin B_6-deficient rats resulted in a further decrease in hippuric acid labeling. The increase in labeled hippuric acid excreted by vitamin B_6-deficient rats injected with glycine-1-^{14}C was not significant in this study although it was in a previously re-

TABLE 1

Effect of vitamin B_6 deficiency on glycine content of rat tissues at different stages of deficiency

Days on diets		+ B_6	− B_6	*P*
		μg glycine/g or ml		
1	Liver	168 ± 20 [1]	177 ± 20 [1]	ns [2]
3		157 ± 14	167 ± 39	ns
6		150 ± 14	153 ± 7	ns
10		261 ± 22	298 ± 34	ns
15		216 ± 10	274 ± 21	< 0.05
1	Kidney	327 ± 19	361 ± 11	ns
3		403 ± 20	419 ± 3	ns
6		466 ± 15	430 ± 14	ns
10		452 ± 16	595 ± 47	< 0.02
15		318 ± 24	483 ± 12	< 0.001
1	Blood	32.7 ± 0.8	31.9 ± 1.1	ns
3		27.4 ± 1.8	25.4 ± 2.1	ns
6		28.2 ± 1.0	27.9 ± 1.6	ns
10		31.2 ± 3.2	44.2 ± 4.4	< 0.05
15		32.0 ± 1.5	40.4 ± 1.2	< 0.01
1	Muscle	327 ± 18	285 ± 15	ns
3		255 ± 15	224 ± 32	ns
6		301 ± 28	422 ± 10	< 0.01
10		422 ± 46	680 ± 32	< 0.01
15		289 ± 28	493 ± 28	< 0.001

[1] Values represent the mean ± SE of duplicate samples from six rats.
[2] ns = not significant.

TABLE 2

Effect of vitamin B_6 deficiency and deoxypyridoxine on the glycine content of rat tissues

Diet	Liver	Kidney	Blood	Muscle	Liver [1]
			μg glycine/g or ml		
+ B_6	157 ± 16	308 ± 24	18 ± 2	167 ± 22	173 ± 6
− B_6	217 ± 37	417 ± 23	20 ± 2	346 ± 37	185 ± 11
Deoxypyridoxine	138 ± 19	285 ± 19	16 ± 2	319 ± 15	121 ± 8

[1] Nine rats per group receiving the experimental diets for 11 days. All other values were obtained from groups of six rats fed the diets 8 days.

ported study of vitamin B_6 deficiency of longer duration (4). However, deoxypyridoxine caused a significant decrease in hippuric acid labeling from glycine-1-^{14}C.

When glycine-1-^{14}C was injected into rats, significantly less respiratory $^{14}CO_2$ was excreted by vitamin B_6-deficient rats than by controls, as seen in table 4. The feeding of deoxypyridoxine to vitamin B_6-deficient rats had no further effect on $^{14}CO_2$ production from labeled glycine in vivo.

In vitro experiments. The oxidation of glycine-1-^{14}C to $^{14}CO_2$ by liver and kidney homogenates was studied with and without the addition of pyridoxal-5-phosphate to the media. As shown in table 5 when pyridoxal phosphate was omitted from the media the oxidation of glycine by liver homogenates from vitamin B_6-deficient rats was depressed. The feeding of deoxypyridoxine to vitamin B_6-deficient rats, however, significantly increased the ability of homogenates of their livers to oxidize glycine. When pyridoxal phosphate was added to the media, glycine oxidation was markedly increased in liver homogenates from all three groups, and homogenates from all three groups oxidized about the same amount of glycine.

Kidney homogenates from both vitamin B_6-deficient and deoxypyridoxine-treated rats oxidized significantly less glycine than did control kidney homogenates when pyridoxal phosphate was omited from the media. Addition of pyridoxal phosphate increased the activity of kidney homogenates from both deficient groups, but not to control values. The ability of kidney homogenates from control rats to oxidize glycine was not affected by the addition of pyridoxal phosphate.

DISCUSSION

The data presented provide further evidence that the metabolism of glycine and some of its associated metabolites is markedly affected by vitamin B_6 deficiency. Variations in tissue levels of glycine may reflect alterations in the synthesis, or catabolism, or both, of this amino acid. As in the report of Swendseid et al. (3), vitamin B_6 deficiency in rats resulted in an increase in tissue glycine levels. In the present study glycine determinations were obtained at different times shortly after the animals were placed on the experimental diets. There appeared to be differences in the time it took for the tissues studied to show significant rises in glycine content, with the process occurring most rapidly in muscle and least rapidly in liver. It cannot be assumed that the feeding of deoxypyridoxine to vitamin B_6-deficient animals merely intensifies the deficiency state. In fact, under certain conditions the analogue appears to reverse some of the effects of vitamin B_6 deficiency (10, 11). In the present study the feeding of deoxypyridoxine did not increase the glycine content of the tissues of rats fed the experimental diet for 8 days. In the case of liver and kidney, but not muscle, it prevented the rise in glycine content in vitamin B_6-deficient rats. In rats fed the diets for 11 days there was significantly less glycine in the livers (the only tissue studied) of vitamin B_6-deficient rats receiving deoxypyridoxine than in the livers of their controls.

The liver is the major hippuric acid synthesizing organ in rats. The data of table 3 and of a previously published study (4) indicate that when vitamin B_6-deficient

TABLE 3

Effect of vitamin B_6 deficiency and deoxypyridoxine on the conversion of labeled compounds to urinary ^{14}C-hippuric acid

^{14}C-compound injected	% of compound excreted as ^{14}C-hippuric acid		
	$+B_6$	$-B_6$	Deoxypyridoxine
Glycine-1-^{14}C	9.05 ± 0.70 [1]	11.16 ± 0.95 [1]	7.89 ± 0.42 [1]
Calcium glycolate-1-^{14}C	15.90 ± 2.50	7.80 ± 0.50	3.50 ± 0.40
Sodium glyoxylate-1-^{14}C	10.20 ± 1.10	4.30 ± 0.50	3.10 ± 0.10

[1] Values represent the mean ± SE of excretions of four groups of three rats fed the experimental diets 5 to 9 days.

TABLE 4

Effect of vitamin B_6 deficiency and deoxypyridoxine on the conversion of glycine-1-^{14}C to $^{14}CO_2$ in vivo

Diet	Injected glycine-1-^{14}C expired as $^{14}CO_2$ per hour
	%
$+B_6$	9.86 ± 0.71 [1]
$-B_6$	6.14 ± 1.02
Deoxypyridoxine	5.82 ± 0.51

[1] Values represent the mean ± SE for eight rats fed the diets for 12 days. The deoxypyridoxine-treated rats were fed a vitamin B_6-deficient diet for 7 days, then the same diet with deoxypyridoxine added for 5 days. Respiratory $^{14}CO_2$ was collected for 1 hour after injection of glycine-1-^{14}C.

rats are injected with labeled glyoxylate or glycolate, which are glycine precursors, less labeled hippuric acid is excreted than by control rats. The opposite is true when labeled glycine is administered to control and vitamin B_6-deficient animals. This may be interpreted as indicating that the activity of the pyridoxal-containing enzymes responsible for the interconversion of glycine and its two carbon precursors is decreased in vitamin B_6 deficiency. In the current study rats receiving deoxypyridoxine converted less labeled glycine, glyoxylate and glycolate to labeled hippuric acid than vitamin B_6-deficient rats not receiving the analogue. Both this observation and the decreased glycine observed in the livers of vitamin B_6-deficient, deoxypyridoxine-treated rats may have been the result of increased oxidation of glycine by liver, as reflected in the increased oxidation of glycine by liver homogenates from deoxypyridoxine-treated rats (table 5).

When vitamin B_6-deficient rats were injected with glycine-1-^{14}C, less $^{14}CO_2$ was expired than by controls. This may have been due to decreased catabolism as observed in vitro with kidney and liver homogenates, but might have also been a reflection of an increased glycine pool in vitamin B_6- deficient rats. The feeding of deoxypyridoxine to vitamin B_6-deficient rats neither reversed nor accentuated the inhibition of total in vivo glycine oxidation caused by vitamin B_6 deficiency.

The oxidation of glycine by rat liver and kidney homogenates in these studies was quite different. When pyridoxal phosphate was not added to the incubation media, the decreased ability of liver homogenates from deficient rats to oxidize glycine was clearly not seen in liver homogenates from deficient rats receiving deoxypyridoxine. Thus, it appeared that the feeding of deoxypyridoxine to vitamin B_6-deficient rats, instead of accentuating the decreased ability of their livers to oxidize glycine, prevented it. This was not seen in studies of kidney homogenates from these same animals. In these studies, there was a similar marked decrease in the ability of kidney homogenates from deficient rats receiving and not receiving deoxypyridoxine to oxidize glycine.

The increased glycine catabolism by liver homogenates from deoxypyridoxine-treated rats may be a result of displacement of pyridoxal phosphate from some enzymes, making it available for others. Sandman and Snell (12) used such an explanation in discussing the vitamin B_6 activity of omega-methylpyridoxine, and Rosen et al. (11) used this explanation for the effect of deoxypyridoxine in reversing the anemia of vitamin B_6 deficiency in dogs. The administration of deoxypyridoxine to vitamin B_6-deficient dogs (11) and rodents (13) has been reported to increase

TABLE 5

Effect of vitamin B_6 deficiency and deoxypyridoxine on the conversion of glycine-1-^{14}C to $^{14}CO_2$ in vitro

Tissue	PALP (0.001 M)	$+B_6$	$-B_6$	Deoxypyridoxine
		μmoles $^{14}CO_2$/hr per mg protein		
Liver	−	17.3 ± 0.9 [1]	14.2 ± 0.9 [1]	21.4 ± 2.1 [1]
Liver	+	59.2 ± 8.1	57.5 ± 2.9	47.7 ± 6.3
Kidney	−	34.4 ± 2.2	12.1 ± 1.6	14.5 ± 0.8
Kidney	+	30.0 ± 2.3	24.1 ± 1.2	20.2 ± 1.4

[1] Values represent the mean ± SE of duplicate samples from six rats. Each sample contained 15 μmoles of substrate in a volume of 3 ml. Rats were fed the experimental diets for 5 to 7 days.

their liver levels of vitamin B_6. Another possibility is that deoxypyridoxine, after conversion to the 5′-phosphate, stabilizes an enzyme, thereby performing a function normally performed by pyridoxal phosphate. Thus, a protective effect may be exerted by deoxypyridoxine similar to the effect reported for it on aspartate beta-decarboxylase (14).

The decreased conversion of glycine to CO_2 by kidney homogenates from vitamin B_6-deficient and deoxypyridoxine-treated rats indicates that pyridoxal phosphate is important in the metabolism of glycine by this organ, as well as by the liver. The failure of added pyridoxal phosphate to restore glycine oxidation to control levels in kidney homogenates from vitamin B_6-deficient and deoxypyridoxine-treated rats indicates a decrease in the protein moiety of the kidney enzymes under the conditions of these experiments.

The fact that the addition of pyridoxal phosphate to liver homogenates from control rats markedly increased their ability to oxidize glycine, but that pyridoxal phosphate did not stimulate glycine oxidation by kidney homogenates from the same animals, suggests that the enzymes involved in glycine metabolism by kidney and liver may be quite different.

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LITERATURE CITED

1. Gershoff, S. N., and F. F. Faragalla 1959 Endogenous oxalate synthesis and glycine, serine, deoxypyridoxine interrelationships in vitamin B_6-deficient rats. J. Biol. Chem., *234:* 2391.
2. Gershoff, S. N., T. J. Gill, III., S. J. Simonian and A. I. Steinberg 1968 Some effects of amino acid deficiencies on antibody formation in the rat. J. Nutr., *95:* 184.
3. Swendseid, M. E., J. Villalobos and B. Friedrich 1964 Free amino acids in plasma and tissues of rats fed a vitamin B_6-deficient diet. J. Nutr., *82:* 206.
4. Runyan, T. J., and S. N. Gershoff 1965 The effect of vitamin B_6 deficiency in rats on the metabolism of oxalic acid precursors. J. Biol. Chem., *240:* 1889.
5. Stekol, J. A., S. Weiss, P. Smith and K. Weiss 1953 The synthesis of choline and creatine in rats under various dietary conditions. J. Biol. Chem., *201:* 299.
6. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138:* 459.
7. Buhler, D. R. 1962 A simple scintillation counting technique for assaying $C^{14}O_2$ in a Warburg flask. Anal. Biochem., *4:* 413.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265.
9. Alexander, B., G. Landwehr and A. M. Seligman 1945 A specific micromethod for the colorimetric determination of glycine in blood and urine. J. Biol. Chem., *160:* 51.
10. Beaton, J. R., M. E. Goodwin, G. Ozawa and E. W. McHenry 1954 The effects of vitamin B_6 and deoxypyridoxine on urea formation in rats. Arch. Biochem. Biophys., *51:* 94.
11. Rosen, F., E. Mihich and C. A. Nichol 1964 Selective metabolic and chemotherapeutic effects of vitamin B_6 antimetabolites. Vitamins Hormones, *22:* 609.
12. Sandman, R., and E. E. Snell 1955 Some effects of omega-methyl analogues of vitamin B_6 in rats. Proc. Soc. Exp. Biol. Med., *90:* 63.
13. Stoerk, H. C. 1950 Deoxypyridoxine observations in "acute pyridoxine deficiency." Ann. N. Y. Acad. Sci., *52:* 1302.
14. Novogrodsky, A., and A. Meister 1964 Protective effect of 4′-deoxypyridoxine 5′-phosphate on aspartate beta-decarboxylase: Evidence for an additional function of vitamin B_6. Biochim. Biophys. Acta, *85:* 170.

Comparative Effects of Gallotannic Acid and Related Phenolics on the Growth of Rats [1,2]

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ABSTRACT The effects of two types of gallotannic acid, of three types of condensed tannins and of gallic acid, ellagic acid and catechin were compared when fed to weanling rats. These phenolic compounds were compared at dietary levels of 5% and at dietary levels ranging from 2 to 10%. Mortality was greatest with galloyl glucose compounds and least with gallic acid, but it was not related to growth depression. Growth depression by condensed tannins was less than that caused by galloyl glucose. Gallic acid and catechin depressed growth but ellagic acid was without any effect.

Most plant phenolic compounds exhibit either no toxic properties to animals, or only a low order of toxicity even in massive oral doses (1), but tannins occurring in carob meal, grain sorghums, lespedeza forage, shin oak bark and other tissue have been reported to depress growth and cause toxicity to cattle, rabbits, rats and chicks. The metabolism of a wide variety of phenolic compounds in animals has been reviewed recently by Williams (2) and De Eds (3). Though chemically defined phenolics have been used in metabolic investigations, tannins have been implicated in feeds only indirectly by analytical procedures of doubtful specificity. Very rarely have the tannins actually present been isolated, characterized and fed to experimental animals. Usually unpurified commercial "tannic acid" is used in supporting evidence. This was true in the early investigations of Lease and Mitchell (4) and Mueller (5), and the more recent investigations of Bornstein et al. (6) and Chang and Fuller (7). Only Camp et al. (8) and Booth and Bell (9) fed the actually isolated and purified tannin.

The commercially available tannins may be either hydrolyzable or condensed tannins. The hydrolyzable Chinese tannins, the so-called gallotannins, are a mixture of gallic acid, *m*-digallic acid, trigallic acid and galloylated glucose; taratannin essentially is a polygalloyl ester of quinic acid; and ellagitannins contain ellagic acid instead of gallic acid (10). The heterogeneity of gallotannins has long been recognized and only recently has the structure of the more commonly available gallotannins been elucidated (11). The composition and structure of condensed tannins still remain to be molecularly defined but these are known to be condensed catechins, leucoanthocyanidins, or mixtures of the two (10, 12).

In the earlier literature there was considerable confusion as to the nature of substances designated as "tannin." Thus Valaer (13), in referring to tannic acid, stated that "tannic acid, gallotannic acid, tannin and digallic acid are terms used interchangeably for the same substance," and cited the United States Pharmacopoeia as authority for this. In the 17th revision of this reference work, published in 1965, tannic acid is described only in the Reagent, Indicators and Solutions section in the most general terms. Rose and Rose (14), however, describe tannic acid as Valaer did. In the Merck Index of Chemicals and Drugs (ed. 7, 1960), it is stated that "digallic acid has been used to denote tannic acid" (p. 356) but on p. 1010 attention is called to the fact that "tannin,

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gallotannic acid, is incorrectly termed digallic acid." The source of tannin and its chromatographic degree of purity should be known to adequately define the preparation used.

Comparison of the growth-depressing effect and toxicity of the wide variety of vegetable tannins has rarely been made for the same test animal. Vohra et al. (15) compared gallotannic acid with several condensed tannins and related phenolics for their growth-depressing effect on chicks. We report a comparison of the toxic properties of several tannins and related polyphenols fed to rats at varying and fixed dietary levels to relate these properties to the composition and structure of the phenolics studied.

MATERIALS AND METHODS

All rats used were males of the Long-Evans strain weighing 50 to 70 g, caged individually, and given food and water ad libitum. In each experiment rats were divided into groups containing 5 to 10 rats/group. Food intake per rat was measured daily, and weights were recorded every 3 to 4 days.

The basal diet composition is shown in table 1. It was prepared by mixing the seven components in sufficient quantities to provide feed for 4 weeks, and stored at 5°. Tannins were added as indicated in the particular feeding experiment, thoroughly mixed with the basal diet powder. The data were analyzed statistically and their significance was established by Student's t test. The phenolics used were the following: chestnut oak bark extract tannin; [4,5] D-catechin, crystalline; [6] ellagic acid, crystalline; [7] gallic acid, crystalline; [8] grape seed tannin [9] (Newmarks' enotannin from Australia); [10] quebracho extract tannin; [11,12] tannic acid, amorphous light brown powder, source unknown, chromatographically impure; [13,14] taratannic acid, light brown colored powder, major constituent penta galloylated quinic acid; [15,16] and wattle bark extract tannin.[17,18]

TABLE 1
Composition of the basal diet

	% wt of diet
Vitamin-free casein [1]	20.00
Cotton seed oil	5.00
Jones-Foster salt mixture (100)	4.00
Choline bitartarate	0.18
Vitamin A, D, E mix	1.00
Vitamin B mix	1.00
Sucrose powder	68.82
Total	100.00

Composition of vitamin B mix

	mg/500 g mix
Thiamine·HCl	200.0
Riboflavin	300.0
Nicotinamide	2250.0
Ca D-pantothenate	1500.0
Pyridoxine·HCl	350.0
Folic acid	100.0
D-Biotin	50.0
Vitamin B_{12} (or 1500 mg of 0.1% triturate)	1.5
Menadione (vitamin K)	50.0
Granulated sucrose	493.7
Total	500.0

Composition of vitamin A, D, E mix

	units/g	*g/1000 g*
Vitamin A distillate	500,000	2.00
Vitamin D_2 (irradiated ergosterol)	400,000	0.25
Tocopherol acetate [1]		5.00
Cotton seed oil		992.75
Total		1000.00

[1] Obtained from General Biochemicals Inc., Chagrin Falls, Ohio.

RESULTS AND DISCUSSION

The effect of these substances on growth of the rats is shown in figures 1, 2 and 3. As shown in figure 1, tannic acid decreased growth at all levels, the decrease being greater at higher levels. At 6% and above the rats actually lost weight and reached initial weight only after 6 to 20 days, except at the 10% level where they all died. Both catechin and gallic acid also depressed

[4] Hydrolyzable tannins. The Folin-Denis reactive phenolic content of the chestnut oak bark extract is given in Vohra et al. (15).
[5] Eastern Regional Research Laboratory, Philadelphia, Penna., 1952.
[6] Mann Research Laboratory, L 2360, New York.
[7] K & K Laboratories, Plainsville, N. Y.
[8] Mann Research Laboratory, L 2203, New York.
[9] Condensed tannins. The Folin-Denis and vanillin phenolic content of these tannins except for the grape tannin are shown in Vohra et al. (15). The phenolic reactive groups of Newmark's grape tannin are given in Joslyn and Dittmar (16).
[10] McKesson, Langley, Michaels Company, San Francisco, 1934.
[11] See footnote 9.
[12] See footnote 5.
[13] See footnote 4.
[14] Baker analyzed reagent grade, gallotannic acid powder, J. T. Baker Chemical Company, Phillipsburg, N. J.
[15] See footnote 4.
[16] Mallinckrodt tannic acid T-8, Mallinckrodt Chemical Works, St. Louis, Mo.
[17] See footnote 9.
[18] See footnote 5.

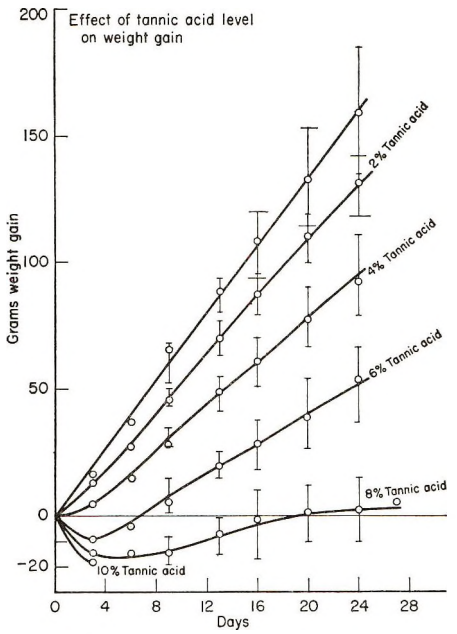

Fig. 1 Effect of level of tannic acid in diet on weight gain.

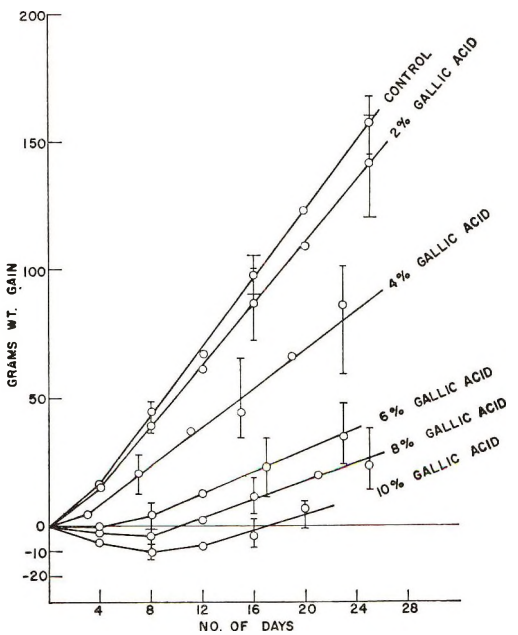

Fig. 2 Effect of level of gallic acid in diet on weight gain.

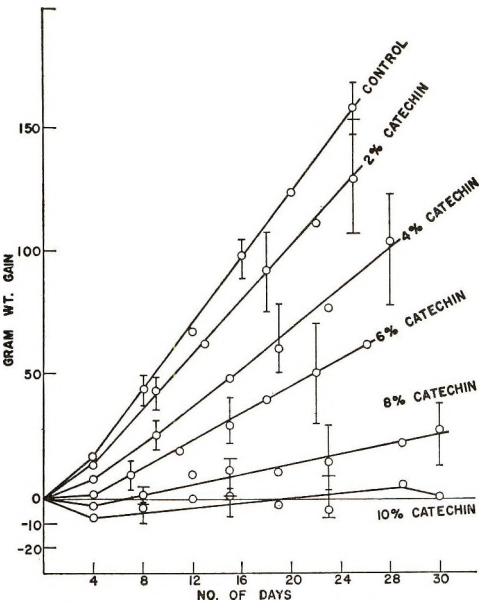

Fig. 3 Effect of level of D-catechin in diet on weight gain.

growth rate but not as markedly as tannic acid (figs. 2 and 3).

Comparative studies of the toxicity of tannic acid, gallic acid, and D-catechin at dietary levels of 2 to 10% showed tannic acid to cause a higher mortality within a shorter period of time at dietary levels of 5, 8 and 10% (table 2).

A true comparison between the toxic effects of tannic acid and those of the other two phenolics could not be obtained from these experiments because the initial weight of the rats on the tannic acid-containing diet was 70 g compared with the 50-g weight of those rats fed the gallic acid and catechin diets. The tolerance of rats to tannic acid was found to depend greatly on their initial weight (as will be discussed in a later report) with the heavier rats having a higher tolerance. This would invalidate a comparison of toxicity where gallic acid and catechin were shown to be more toxic than tannic acid on the one hand, yet would further verify the observation that tannic acid was more toxic at 5, 8 and 10% levels.

The toxic properties of these three phenolics were later compared by feeding at a 5% dietary level to weanling rats (table 3). The average weight gain of the rats

TABLE 2

Effect of tannic acid, gallic acid, and D-*catechin at 2 to 10% dietary levels on rat mortality*

Treatment	No. of rats	Dietary level	Occurrence of death, days from the beginning of feeding	Total deaths
		%		
Tannic acid	10	2		0
	10	4		0
	10	5	5 to 8	3
	10	6		0
	10	8	4 to 7	7
	10	10	4 to 5	10
Gallic acid	10	2		0
	10	4		0
	5	5		0
	10	6	12	1
	10	8	12 and 15	2
	10	10	10 to 19	6
D-Catechin	10	2		0
	10	4	10	1
	5	5		0
	10	6	15	2
	10	8	7 to 20	4
	10	10	6 to 27	7

TABLE 3

Effect of various phenolics on weight gain, feed intake and mortality

Treatment	No. of rats	No. of deaths	No. of days	Avg wt gain per rat per day	Avg food intake per rat per day
				g	*g*
Control	15	0	21	5.2 ± 0.8 [1]	13.8 ± 1.5 [1]
5% tannic acid	5	2	21	1.1 ± 0.2 ***	7.9 ± 0.2
5% taratannic acid	10	0	21	2.4 ± 0.4 ***	9.9 ± 0.8
5% gallic acid	5	0	18	0.6 ± 0.2 ***	7.9 ± 1.2
5% D-catechin	5	0	18	1.4 ± 0.7 ***	9.6 ± 1.6

[1] ± SD.
*** Significant at $P < 0.001$.

fed various tannins is shown in figures 4 and 5. Growth depression caused by the various phenolic compounds did not necessarily coincide with mortality (tables 3 and 4). Gallic acid had a higher growth depressing effect at 4 and 6% (in weeks 2 and 3) and at a 5% dietary level than did tannic acid, yet a higher mortality occurred with the tannic acid but not with the gallic acid. Food intake was the same in the gallic acid group as in the tannic acid group (table 3). A similar observation on food intake was found when a 6% dietary level of each of these two phenolics was fed, but not at other levels (table 5).

D-Catechin had a lower growth and food intake-depressing effect than tannic acid (tables 3 and 5). No mortalities occurred at a 5% level of catechin, whereas two out of five rats died under the same treatment with tannic acid (table 3). Earlier results (table 2) showed that 1 rat out of 10 died at a 4% level and 2 out of 10 rats at a 6% dietary level of D-catechin. Thus, D-catechin appeared to show lethal effects more similar to tannic acid than does gallic acid.

When taratannic acid, in which quinic acid replaces the glucose of gallotannic acid, was fed at a 5% dietary level (table 3), it alleviated to some extent the growth-depressing properties. Growth, however, was still less than 50% that of the control.

A comparative study on the effects of tannic acid, ellagic acid, grape seed tan-

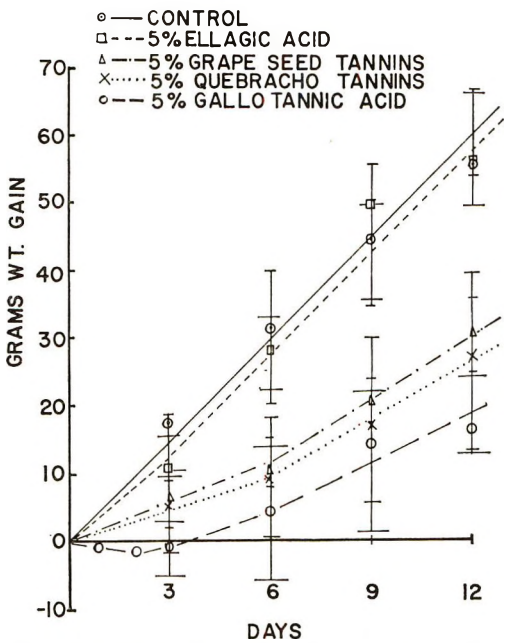

Fig. 4 Effect of ellagic acid, grape seed tannin, quebracho tannin and gallotannic acid at 5% level on weight gain.

nins and quebracho tannins at 5% dietary levels on growth of rats is shown in figures 4 and 5. Ellagic acid (which is the condensation product of two gallic acid molecules in which each carboxyl group is bonded forming a depside with the meta hydroxyl of the adjacent molecule) was completely devoid of any of the deleterious effects of any of the other polyphenolics tested, both in regard to growth and to feed intake (table 6). This observation may suggest that

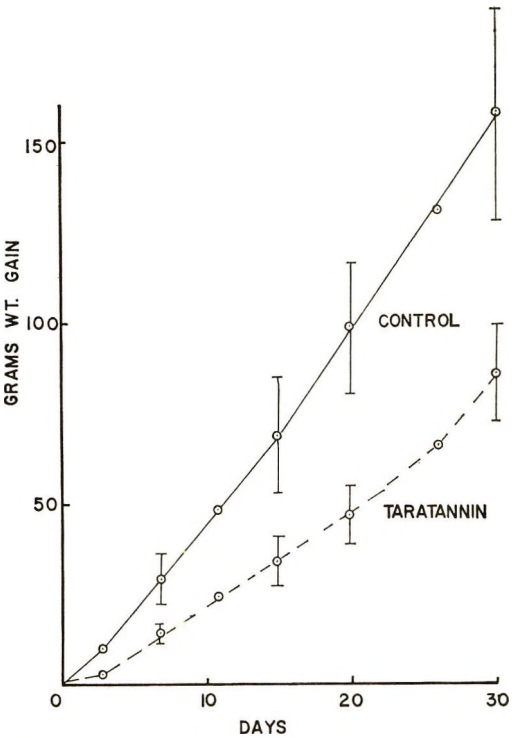

Fig. 5 Effect of taratannin at 5% level on weight gain.

TABLE 4

Effect of the type and level of phenolic on growth of rats

Phenolic	No. of rats (survivors)	Dietary level	Growth depression (expressed as percentage growth of control)		
			1 week	2 weeks	3 weeks
		%			
Tannic acid	10		78	81	82 **
Gallic acid	10	2	89	88	89 *
D-Catechin	10		85	82	83 **
Tannic acid	10		48	56	58 ***
Gallic acid	10	4	56	55	55 ***
D-Catechin	9		50	52	55 ***
Tannic acid	10		8	24	31
Gallic acid	9	6	7	20	24
D-Catechin	8		25	34	37
Tannic acid	3		−30	−13	4.4
Gallic acid	8	8	−11	7	15
D-Catechin	6		0	8	11

* Significant at $P < 0.05$.
** Significant at $P < 0.01$.
*** Significant at $P < 0.001$.

TABLE 5

Effect of the type and level of phenolic on feed consumption (expressed as percentage food intake of control)

Phenolic level	No. of rats	Tannic acid	No. of rats	Gallic acid	No. of rats	D-Catechin
2	10	85	10	103	10	83
4	9	68	10	89	7	85
6	8	58	9	58	8	64
8	3	43	6	57	6	54
10	—	—	4	49	2	44

TABLE 6

Effect of various polyphenolics on mortality, growth and feed consumption of rat when fed at 5% level

Treatment	No. of rats	No. of deaths	No. of days	Avg wt gain per rat per day	Avg food intake per rat per day	Growth depression (expressed as percentage growth from control)
				g	*g*	
Control	5	0	12	4.65 ± 0.63 [1]	10.1 ± 1.1 [1]	100
5% tannic acid	5	1	12	1.37 ± 0.48 *	7.9 ± 2.0	30
5% ellagic acid	5	0	12	4.69 ± 0.73 **	11.3 ± 1.2	101
5% quebracho acid	5	0	12	2.26 ± 1.10 *	7.7 ± 1.4	48
5% grape seed tannins	5	0	12	2.56 ± 0.43 *	8.1 ± 0.9	55

[1] ± SD.
* Significant at $P < 0.001$.
** Not significant.

TABLE 7

Effect of various polyphenolics on growth and food intake of rats when fed at 1% level

Treatment	Control	Tannic acid	Chestnut oak bark	Quebracho extract	Wattle bark extract
No. of rats	9.00	10.00	9.00	10.00	10.00
No. of days	32.00	32.00	32.00	32.00	31.00
Avg wt gain,	6.30	5.30 *	5.70 **	5.90 **	6.10 **
g per rat per day	± 0.75 [1]	± 0.79	± 0.47	± 0.46	± 0.75
Avg food intake,	15.50	13.80	14.60	15.00	15.80
g per rat per day	± 1.88 [1]	± 1.37	± 0.94	± 0.92	± 0.92

[1] ± SD.
* Significant at $P < 0.02$.
** Not significant.

ellagic acid is not broken down into its gallic acid components in the gastrointestinal tract, or the toxic properties of gallic acid would have been revealed. It is also possible that if the sole effect of gallic acid is on feed palatability, ellagic acid, which is obviously palatable, even if it decomposes into its gallic acid components, will be nontoxic. The latter possibility is made feasible by data showing that intraperitoneal injections of gallic acid are nontoxic (17).

The condensed tannins from quebracho and grape seed showed a significant growth and feed intake depression (fig. 4, table 6). The extent of growth depression caused by a 5% level of quebracho or grape seed tannins (table 6) is of the same order as that caused by 4% catechin (table 4), i.e.: 55, 48 and 52% of controls for the grape seed

tannins, quebracho tannins and catechin, respectively, suggesting that condensation of the catechin moiety reduces its growth-depressing properties.

A comparative study of the effects of tannic acid, chestnut oak bark extract, quebracho extract and wattle bark extract tannins given at a 1% dietary level (table 7), show tannic acid to be most effective as a growth and food intake depressant. Although our data on the effect of a 1% dietary level of tannic acid agree closely with the results of analogous feeding to chicks (15), higher levels of this compound, i.e., 3 to 7%, caused a higher mortality in chicks than did similar levels in rats, suggesting that chicks are more susceptible in their tolerance to the tannic acid diet.

DISCUSSION

Our results are similar to the earlier data reported by Lease and Mitchell (4) who found that adult albino rats tolerated 5% crystalline tannic acid (digallic acid?) whereas higher levels were toxic. They observed gallic acid to be more toxic than tannic acid but did not give the data on which this conclusion was based. Mueller (5) found that even 2% crystalline tannic acid depressed growth of rats. On the other hand, Booth and Bell (9) reported that sericea tannin could be fed to albino rats at 28 days of age for periods of 121 to 150 days without any effect on mean body weight gain or feed efficiency (weight gain per gram feed intakes). Grape pomace phlobatannin, similar to the grape seed tannin we used, was fed to rats at a 2% level for 400 to 700 days without any significant effect. With sericea tannin, Booth and Bell (9) did not detect any phenolic degradation product in rat urine and concluded that little if any absorption of the tannin occurred. With chicks Vohra et al. (15) reported that tannic acid at a level as low as 0.5% depressed growth significantly. At 1% level, wattle bark depressed growth more than tannic acid, and quebracho tannin had no effect after 7 days feeding, but after 20 days, wattle bark and quebracho were similar and tannic acid considerably more toxic.

Though much of the decrease in growth was related to a decrease in food intake, which with tannic acid and gallic acid at the 5% level was about 50% of the control and decreased with increase in level from 2% to 10%, other factors also were involved. It will be reported in succeeding papers that the phenolic reduced absorption of dietary components and in addition exerted a still unknown toxic effect.

LITERATURE CITED

1. Whalley, B. 1959 The toxicity of plant phenolics. In: The Pharmacology of Plant Phenolics, ed., J. W. Fairbairn. Academic Press, New York, p. 27.
2. Williams, R. J. 1964 Metabolism of phenolics in animals. In: Biochemistry of Phenolic Compounds, ed., J. B. Harborne. Academic Press, New York, p. 205.
3. De Eds, F. 1968 Flavonoid metabolism. In: Comprehensive Biochemistry, vol. 20, Metabolism of Cyclic Compounds, eds., M. Florkin and E. H. Stotz. Elsevier Publishing Company, New York, p. 127.
4. Lease, E. J., and J. H. Mitchell 1940 A study on the tannins of *Lespedeza sericea*. S. C. Agr. Exp. Sta. Ann. Rep., *53:* 710.
5. Mueller, W. S. 1942 The significance of tannic substances and theobromine in chocolate milk. J. Dairy Sci., 25: 221.
6. Bornstein, S., E. Alumot, S. Mokadi, E. Nachtomi and U. Nahari 1963 Trials for improving the nutritional value of carobs for chicks. Israeli J. Agr. Res., *13:* 25.
7. Chang, S. I., and H. L. Fuller 1964 Effect of tannin content of grain sorghums on their feeding value for growing chicks. Poultry Sci., *43:* 30.
8. Camp, B. J., E. Steel and J. W. Dallahite 1967 Certain biochemical changes in blood and livers of rabbits fed oak tannins. Amer. J. Vet. Res., 28: 290.
9. Booth, A. N., and T. A. Bell 1968 Physiological effects of sericea tannin in rats. Proc. Soc. Exp. Biol. Med., *128:* 800.
10. Haslam, E. 1966 Chemistry of Vegetable Tannins. Academic Press, New York.
11. Haslam, E. 1967 Gallotannins. Part XIV. Structure of the gallotannins. J. Chem. Soc., *1967:* 1734.
12. Harborne, J. B. 1967 Comparative Biochemistry of the Flavonoids. Academic Press, New York, p. 97.
13. Valaer, P. 1941 Report on whisky and rum. Tannins in potable spirits. J. Assoc. Offic. Agr. Chem., 24: 224.
14. Rose, A., and E. Rose 1961 The Condensed Chemical Dictionary, ed. 6. Reinhold Publishing Company, New York, p. 1105.
15. Vohra, P., F. H. Kratzer and M. A. Joslyn 1966 The growth depressing and toxic effects of tannins to chicks. Poultry Sci., 45: 135.

16. Joslyn, M. A., and H. F. K. Dittmar 1967 The proanthocyanidins of Pinot blanc grapes. J. Enol. Vitic., *18:* 1.

17. Clark, E. G. C., and E. Cotchin 1956 A note on the toxicity of the acorn. Brit. Vet. J., *112:* 135.

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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*)
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square meter	m^2
square millimeter	mm^2
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standard error	SE
standard error of the mean	SEM
t (statistics)	*t*
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ERRATUM

Ross, Morris H. 1969 Aging, nutrition and hepatic enzyme activity patterns in the rat. J. Nutr., *97:* 563. On page 569, column 2, following line 11, the cube root sign is missing in the formula.

To correct this error in your copy of volume 97, number 4, supplement 1, page 569, please cut along the lines of reprinted section below and paste over the incorrect formula immediately following line 11, column 2.

$$Pb_1 = \frac{D}{\sqrt[3]{\frac{3V}{4\pi}} + \frac{1}{2}T}$$
