A Semipurified Diet for the Mongolian Gerbil (*Meriones unguiculatus*)

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ABSTRACT A semipurified casein-glucose diet was developed for use as a basis for further work on nutritional requirements of the Mongolian gerbil. It supports a growth rate equal or superior to that obtained from a commercial pelleted diet in common use. Animals fed this diet also appear to be normal with respect to appearance, physical activity, hematological values, and relative organ size. Increasing the protein, vitamin, or mineral content of this semipurified diet did not improve growth rate or cause significant changes in the other parameters measured.

The Mongolian gerbil, *Meriones unguiculatus*, is now being used more frequently as a laboratory animal. As such, it has a number of attributes which promise to make it useful as an experimental animal and has already been used in studies on such subjects as cholesterol metabolism (1–4) [1] and tumors (5, 6).

The general characteristics and management of the gerbil have been described by Schwentker (7). It is easily cared for in the laboratory, requiring only small amounts of water and excreting small quantities of highly concentrated urine. Odors are minimal since feed and excreta are relatively dry. Supplies of feed and water for several days or more can be provided, during which time the animal may be left without attention. The gerbil adapts easily to a wide range of diets of natural foods and environmental conditions (7).

A study of its water metabolism has been made by Winkelmann and Getz (8), but there has been no systematic study of its nutritional needs. Therefore, the purpose of the present investigation was to devise a purified diet which could be used as a basis for further nutritional studies with this animal. At present, no work has been published on the composition of such diets or on the nutritional requirements of the gerbil.

MATERIALS AND METHODS

Both male and female weanling Mongolian gerbils were used. Preliminary work resulted in high mortality rates if the gerbils were not allowed a period of adjustment to the new environment. It was also observed that animals housed in wire-bottom cages without bedding developed very rough coats within a few days regardless of the diet used. Consequently all animals, when received, were housed in sterilized cages containing bedding material and fed a stock commercial pelleted rat food until they were gaining weight. At this time, animals that were to be fed a semipurified diet were given the control diet for a period of at least 3 days to give time for adjustment to a mash-type diet. The work was then continued as follows.

Part 1. Gerbils have been reported to thrive on commercial pelleted rat food (7). Therefore, 12 weanlings of each sex were divided into 2 groups. Each group consisted of 6 males and 6 females with a mean weight of 50 g for males and 46 g for females. One group was fed a commercial pelleted rat food,[2] hereafter referred to as "stock diet." The other group was given a semipurified casein-glucose diet in the form of a loose mash, the composition of which is shown in table 1 and listed as the "basic semipurified diet." Both food and water were provided ad libitum.

Animals were weighed twice weekly. At the time of weighing, each animal was also examined grossly for abnormalities. This procedure was followed for 10 weeks. One male in the control group was discarded at

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[1] Gordon, S., and W. P. Cekleniak 1960 Hypercholesteremia and absence of atheroma in the gerbil. Federation Proc., 19: 231 (abstract).

[2] Wayne Lab Blox, Allied Mills, Inc., Chicago.

TABLE 1

Composition of diets

	Basic semipurified diet	35% protein diet	High vitamin diet	High salt diet
	%	%	%	%
"Vitamin-free" casein	30	35	30	30
Corn oil	2	2	2	2
Cellulose [1]	5	5	5	5
Salt mix [2]	6	6	6	8
Vitamin mix [3]	1.5	1.5	3	1.5
Dextrose	55.5	50.5	54.0	53.5

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] Grams per kg of salt mix: $CaCO_3$, 300.0; K_2HPO_4, 325.0; $CaHPO_4$, 60.0; NaCl, 168.0; $FeSO_4 \cdot 7H_2O$, 25.0; $MgSO_4 \cdot 7H_2O$, 125.0; KI, 0.8; $ZnCO_3$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.3; and $MnSO_4 \cdot H_2O$, 2.3.
[3] Grams per kg of vitamin mix: choline chloride, 50.0; inositol, 25.0; ascorbic acid, 5.0; Ca pantothenate, 2.5; *p*-aminobenzoic acid, 0.5; menadione, 1.25; thiamine·HCl, 1.5; riboflavin, 0.5; pyridoxine, 1.5; nicotinic acid, 1.5; biotin, 0.0125; folic acid, 0.03; glucose monohydrate (Cerelose, Corn Products Company, Argo, Illinois), 912; 0.1% crystalline vitamin B_{12} in mannitol, 12 mg; *dl*-α-tocopherol, 80 mg; high potency cod liver oil, (Rexall Drug Company, St. Louis), 6 ml.

the end of 6 weeks because of an upper respiratory infection.

Part 2. There is little information on the growth rate of the gerbil in the literature; therefore, it was not possible to judge on that basis whether either group of animals in part 1 grew at a normal rate. To test the possibility of improved growth rate following further changes in the diet, part 2 of this work was undertaken.

Four groups of animals were established with 5 weanling males and 5 weanling females in each. The sexes were caged separately. Each group had a mean weight of 57 g for males and 42 g for females. The diets were as follows: group 1, which served as the control group, the basic semipurified casein-glucose diet used in part 1 above; groups 2, 3 and 4, semipurified diets as in group 1 adjusted to give increased amounts of protein, vitamins, and minerals, respectively. The composition of these diets is also shown in table 1. Procedures for feeding and weighing animals were the same as in the first series and were followed for 10 weeks.

In addition, in each series, at the end of the experimental period, hematological studies were carried out. Hemoglobin levels, erythrocyte and leukocyte counts, hematocrits, differential counts, and erythrocyte diameters were determined. Each animal was anesthetized with 40 mg of sodium pentobarbital per kg of body weight, and blood was obtained by amputation of the tip of the tail. Total erythrocyte and leukocyte counts were made following the usual clinical procedures (9) with Hayem's solution and 1% acetic acid as the respective diluents. Smears for differential counts were stained with Jenner-Giemsa stain, and 100 cells were counted from each of 2 or 3 slides. Hemoglobin was determined by the acid hematin method (10) and hematocrit by centrifuging blood collected into heparinized capillary tubes. Red cell diameter was measured with an ocular micrometer. From the above data, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were calculated.

After blood was obtained, all animals were examined internally for gross anatomical lesions. Liver, kidneys, adrenals, testes, heart, and brain were removed and weighed from all animals in part 1 and all females in part 2.

Results in part 1 were analyzed by Student's *t* test and in part 2 by analysis of variance (11).

RESULTS

Part 1. Results of growth and hematological studies and of determination of organ weight-to-body weight ratios are shown in tables 2 and 3, respectively. No significant differences were found in total growth or in hematological values between the 2 groups. Both males and females fed the stock diet had a liver significantly larger, at the 5% level, in proportion to body weight, than those fed the semipurified diet. There were no significant differences between the 2 groups in relative weights of the other organs considered.

TABLE 2

Weight increase and hematological values of Mongolian gerbils fed two diets

	Males		Females	
	Stock	Semipurified	Stock	Semipurified
10-week wt increase, g	20.6 ± 5.5 [1]	27.0 ± 7.1	14.0 ±4.1	19.9 ± 5.8
Erythrocytes × 10^6/mm^3	9.38± 1.02	8.80± 1.17	9.80±1.25	9.44± 2.51
Erythrocyte diam, μ	6.88± 0.56	6.69± 0.17	7.18±0.51	6.48± 0.33
Hematocrit, %	59.45±12.41	52.05± 4.52	63.2 ±3.55	57.2 ± 5.31
Hemoglobin,g/100 ml	14.6 ± 2.10	15.3 ± 0.88	15.2 ±0.80	14.9 ± 1.10
Mean corpuscular vol, μ^3	63.3 ± 9.00	66.1 ±10.62	65.6 ±6.41	63.2 ±10.57
Mean corpuscular Hb, $\mu\mu$g	15.53± 1.46	18.05± 2.74	15.95±2.94	16.71± 2.88
Mean corpuscular Hb conc, %	27.07± 4.20	29.66± 2.91	24.15±2.45	26.37± 2.24
Leukocytes/mm^3	6992±984	6700±706	6400±3022	7220±3775
Differential count, % :				
lymphocytes	78.25± 7.82	74.67± 9.35	70.8 ±7.25	80.0 ± 7.23
neutrophils	19.92± 8.26	22.67± 8.58	27.30±6.16	18.8 ± 7.73
basophils	0.92± 0.99	0.33± 0.36	1.00±0.818	0.2 ± 0.4
eosinophils	0	0	0	0
monocytes	0.92± 1.49	2.20± 2.83	0.83±0.69	1.00± 1.55

[1] Mean ± one sd.

TABLE 3

Organ weight-body weight ratios [1] of Mongolian gerbils fed two diets

	Males		Females	
	Stock	Basic semipurified	Stock	Basic semipurified
Liver	3.72 ±0.37 [2]	3.05 ±0.12 [3]	3.91 ±0.36	3.32 ±0.22 [3]
Spleen	0.1324±0.3991	0.097 ±0.3010	0.1219±0.0500	0.1747±0.4000
Adrenals	0.0455±0.0080	0.0440±0.0055	0.0460±0.0042	0.0490±0.0062
Kidneys	0.772 ±0.0583	0.736 ±0.0678	0.805 ±0.0742	0.7257±0.0565
Heart	0.365 ±0.035	0.328 ±0.024	0.354 ±0.032	0.3634±0.020
Brain	1.24 ±0.162	1.28 ±0.167	1.6069±0.198	1.56 ±0.076
Testes	1.37 ±0.15	1.32 ±0.13	—	—

[1] Ratios are expressed as percentage of total body weight.
[2] Mean ± one sd.
[3] Significantly different at 5% level of probability.

There were no observable differences in general appearance or activity of the animals in the 2 groups.

Part 2. Analysis of variance of weight increases, shown in table 4, indicates no significant differences in growth response. This fact is strong evidence that there are no real differences among these diets since it would be highly fortuitous if all variations produced exactly the same response.

There were no significant differences among the various groups in hematological values or organ weight-to-body weight ratios. The data are summarized in tables 4 and 5. Also, as in part 1, there were no observable differences in appearance or activity of the animals in the various groups.

Animals were occasionally observed to have a "convulsive" reaction when being handled. The reaction began with a marked blinking of the eyes and twitching of the whiskers. This was usually followed by muscle spasms causing extension of the limbs. The head was often extended abnormally, but occasionally was curled under the body so that the animal turned a somersault. These spasms occurred in less than a minute and were followed by a period when the animals were very quiet.

TABLE 4

Weight increase and hematological values in Mongolian gerbils fed four semipurified diets

	Males				Females			
	Basic semipurified	35% protein	High vitamin	High salt	Basic semipurified	35% protein	High vitamin	High salt
73-day wt increase, g	32.2 ± 7.3	26.2 ± 6.4	26.0 ± 6.1	26.4 ± 5.9	21.2 ± 5.5	20.0 ± 5.3	18.8 ± 4.9	19.0 ± 5.0
Erythrocytes $\times 10^6/mm^3$	8.23 ± 0.85	8.90 ± 1.49	9.13 ± 2.17	8.32 ± 1.63	9.34 ± 2.21	9.55 ± 1.62	9.79 ± 3.05	9.13 ± 1.10
Erythrocyte diam, μ	7.3 ± 0.63	6.9 ± 0.84	6.9 ± 0.36	7.4 ± 0.20	6.8 ± 0.81	7.2 ± 0.75	6.9 ± 0.69	6.9 ± 0.56
Hematocrit, %	59.8 ± 1.8	55.4 ± 4.8	54.0 ± 7.8	57.1 ± 2.6	56.2 ± 5.2	62.4 ± 9.1	63.1 ± 4.3	57.6 ± 2.3
Hb, g/100 ml	15.94 ± 0.40	15.98 ± 0.47	16.14 ± 0.92	16.08 ± 0.17	14.83 ± 1.23	15.50 ± 0.51	15.06 ± 1.31	14.50 ± 0.84
Mean corpuscular vol, μ^3	73.1 ± 8.4	63.7 ± 9.5	60.4 ± 5.8	71.3 ± 16.3	64.3 ± 13.8	66.9 ± 8.5	70.0 ± 18.6	63.9 ± 6.7
Mean corpuscular Hb, $\mu\mu g$	19.5 ± 0.96	18.5 ± 2.99	18.4 ± 2.60	20.0 ± 4.40	16.6 ± 2.93	16.6 ± 1.91	16.5 ± 3.86	16.0 ± 1.62
Mean corpuscular Hb conc, %	26.7 ± 1.50	29.0 ± 1.85	30.2 ± 2.47	28.2 ± 0.55	25.4 ± 1.70	24.8 ± 1.14	23.9 ± 9.98	25.2 ± 1.04
Leukocytes, mm^3	5160 ± 1229	6250 ± 2367	5940 ± 4139	7080 ± 1257	7920 ± 3413	8040 ± 2413	7060 ± 3318	6080 ± 5699
Differential count, % :								
lymphocytes	73.0 ± 8.46	73.5 ± 13.01	71.0 ± 11.24	74.6 ± 10.46	79.6 ± 7.94	83.2 ± 4.12	80.6 ± 8.00	76.0 ± 8.89
neutrophils	24.8 ± 15.6	26.0 ± 13.0	28.6 ± 11.2	25.1 ± 10.5	19.4 ± 7.75	15.6 ± 5.00	18.8 ± 8.37	22.0 ± 9.54
basophils	0.4 ± 0.5	0.4 ± 0.4	0	0	0.2 ± 0.4	0.8 ± 0.4	0.2 ± 0.4	1.75 ± 0.8
eosinophils	0	0	0	0	0	0	0	0
monocytes	1.8 ± 2.76	0.1 ± 0.22	0.4 ± 0.49	0.3 ± 0.48	0.8 ± 1.55	0.4 ± 0.49	0.4 ± 0.80	0.2 ± 0.4

[1] Mean ± one SD.

TABLE 5

Organ weight-body weight ratios [1] in female Mongolian gerbils fed four semipurified diets

	Basic semipurified	35% protein	High vitamin	High salt
Liver	3.22 ±0.24 [2]	3.05 ±0.17	3.15 ±0.22	3.05 ±0.49
Spleen	0.1347±0.0223	0.1006±0.0245	0.1174±0.0469	0.1411±0.0400
Adrenals	0.0450±0.0105	0.0488±0.0084	0.0469±0.0071	0.0468±0.0032
Kidneys	0.7417±0.0800	0.7262±0.0332	0.6991±0.0616	0.7163±0.0520
Heart	0.3532±0.0490	0.3485±0.0141	0.3568±0.0265	0.3381±0.0141
Brain	1.5855±0.0775	1.6222±0.0107	1.5666±0.0933	1.6338±0.0728

[1] Ratios are expressed as percentage of total body weight.
[2] Mean ± one SD.

This phenomenon occurred in some animals, but not all, in each diet group. No relationship could be found between the occurrence of these symptoms and the diet, hematological values, or gross appearance of internal organs.

DISCUSSION

A semipurified diet for the Mongolian gerbil was developed which supports a rate of growth at least equal to or possibly superior to that obtained when the animals are fed a commercial pelleted rat diet. Increasing the protein, vitamin, or mineral content of the diet does not result in significant differences in growth rate, hematological values measured, appearance, or activity.

It is not the intention to imply that this diet might not be improved by other modifications. The maximal growth rate of the gerbil is unknown, as are its nutritional requirements. Therefore, it is possible that further variation in the proportion of presently used nutrients or addition of others which are now unknown might increase the growth rate obtained in this study. However, it is believed that the semipurified diet used in this study can be used as a basis for further studies on the nutritional needs of the Mongolian gerbil.

Hematological values obtained in this study from gerbils approximately 4 months old were compared with those obtained by Ruhren (12) who published values for gerbils at the ages of 2, 7, and 13 months. The mean values in this study were slightly higher for hemoglobin and hematocrit levels and for erythrocyte counts. The leukocyte levels were markedly lower than those determined by Ruhren.

Special note must be made of the absence of eosinophils in the blood smears of all of these animals. Since Ruhren (12) has reported the presence of eosinophils, this is evidently not a species characteristic. Also worthy of note is that no eosinophils were observed in the blood smears of animals having the "convulsive" seizures, described previously. This was true even of the occasional animal which had a seizure immediately before the blood was taken. In mice, mild stress such as that from handling and taking blood results in a great decrease in eosinophils which continues about 7 hours (13). It is possible that the handling of the gerbils might also have resulted in such a decrease, particularly since they were handled very little prior to the time when the blood samples were taken.

No explanation is available for the difference in liver size in animals fed the stock diet in part 1. All livers were normal in gross appearance on postmortem. Livers were not examined histologically.

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Effects of Dietary Protein Level on Growth and Proteolytic Activity of the Avian Pancreas [1,2]

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ABSTRACT Pancreatic size, mitotic activity and protease content were determined in normal and protein-depleted chicks after feeding purified isolated soybean protein-dextrose diets containing zero, 15, 30, 50 and 70% protein. The proteolytic activity and mitotic rate were not affected by feeding 50 and 70% protein diets to normal chicks. When diets containing zero or 15% protein were fed to normal chicks there was a reduction in both proteolytic activity and mitotic rate. The pancreas of the normal chicks, generally, increased in size when the level of dietary protein increased. The feeding of protein-containing diets to depleted chicks resulted in an increased synthesis of proteases, hyperplasia and in an increased size of the pancreas. The amount of proteases was greater with the higher levels of protein for the first 2 days of treatment. Thereafter, the proteolytic activity decreased and the mitotic rate increased. The pancreas of the depleted chicks was larger than that of the normal chicks fed corresponding levels of protein.

The reports of Grossman et al. (1, 2) indicated that the synthesis of enzymes by the pancreas was affected by the nature of the diet. Ben Abdeljlil et al. (3) reported an increased secretion of trypsinogen and chymotrypsinogen by the pancreas of rats fed a 70% casein diet. High protein-low starch diets have been reported by Howard and Yudkin (4) to result in greater protease synthesis by the rat pancreas.

The relative high efficiency in protein utilization during the early stages of protein repletion of malnourished Jamacian infants (5) indicates an increased action of compensatory mechanisms throughout the animal body tending to replace the tissue lost during the period of protein lack. The possibility that the exocrine pancreas is involved in this overall process was suggested in the study by Snook (6) who reported that prior treatment with a nitrogen-free diet resulted in a greater amount of proteolytic activity than was observed in rats fed protein-containing diets without a previous depletion period. Leduc (7) observed an increased mitotic activity in the liver of fasted rats when refed protein-containing diets. The time required for the peak in the mitotic rate to occur varied inversely with the level of protein in the diet.

A study of the possible relationship between protease synthesis and tissue growth in the pancreas of chicks is reported in the present paper. Measurements were made of proteolytic and mitotic activity in the pancreas of normal and protein-depleted chicks when fed diets differing in protein content.

METHODS

Male broiler-type chicks were reared to 17 days of age with an isolated soybean protein [4]-dextrose diet containing 30% protein. The chicks that were transferred from the 30% protein diet to the various dietary levels of protein are referred to as normal chicks. Depleted chicks are those fed a protein-free diet for 10 days prior to the feeding of the various diets.

The protein source in all diets except for the one casein study was isolated soybean protein supplemented with glycine and methionine. The amino acid composition of the protein portion of the diet was constant for all levels of dietary protein. All additions of protein to the diet were made

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[4] C-1 Assay Protein, Skidmore Enterprises, Cincinnati.

at the expense of an equivalent amount of dextrose. The composition of the diets is shown in tables 1–3. Ad libitum feeding was used throughout the study.

At the termination of the treatments, the pancreas of each chick was removed after killing the chicks by cervical dislocation. The pancreas was weighed immediately, then homogenized with a tissue grinder and lyophilized. Proteolytic activity was determined on a 10-mg sample of the lyophilized pancreas after activating the zymogens by the method of Lepkovsky et al. (8). A 2% hemoglobin substrate (9) was used, and the tyrosine liberated during a 10-minute incubation period was determined. Lyophilized chick pancreas (LCP), which was prepared by lyophilizing the pooled homogenized pancreata of a group of 3-week-old chicks fed the 30% isolated soybean protein diet, was used as a reference standard. The proteolytic activity was expressed as the amount (mg) of LCP required to liberate a quantity of tyrosine equal to that liberated by the 10-mg experimental sample.

Colchicine, a drug which arrests mitosis in metaphase, was used to measure the mitotic rate in the pancreatic acinar tissue. The chicks used in this experiment were injected intramuscularly with 0.1 mg colchicine/100 g body weight. Four hours later the chicks were killed, and histological sections of their pancreas were made. The sections were cut at 5 μ and stained with hematoxylin and eosin. A Polyethlene mask was placed in the ocular of a microscope to obtain a rectangular field large enough to encompass about 30 cells at 970 ×. The number of mitotic and interphase cells within the field were counted at 5 locations in the sections. The mitotic rate was expressed as the percentage of cells which entered mitosis during the 4-hour colchicine treatment period.

TABLE 1

Protein-free basal diet fed as 20% of the experimental diets

	g
Salt mixture [1]	252.9
Corn oil	50.0
Choline·Cl	10.0
Vitamin mixture [2]	10.0
Procaine penicillin	0.05
α-Tocopherol	0.50
$MgSO_4$	12.50
$ZnCO_3$	0.50
Dextrose [3]	663.55
Total	1000.00

[1] Composition of the salt mixture in grams: NaCl, 104.20; $CaHPO_4 \cdot 2H_2O$, 473.10; $CaCO_3$, 292.90; KCl, 118.80; $FeSO_4 \cdot 7H_2O$, 6.25; $MnSO_4 \cdot H_2O$, 4.15; $CaSO_4 \cdot 5H_2O$, 0.40; KI, 0.20.
[2] Scott et al. (16).
[3] Cerelose, Corn Products Company, Argo, Illinois.

RESULTS

In experiment 1, a comparison of proteolytic activity and pancreas size was made between chicks fed the 30% protein diet and the protein-free diet during a 10-day period. Five chicks from each group were killed after 1, 2, 4, 7 and 10 days of treat-

TABLE 3

Composition of the casein diets

	Crude protein,[1] %		
	0	30	70
	% of diet		
Protein-free basal [2]	20.0	20.0	20.0
Casein protein [3]	—	34.2	80.0
Dextrose [4]	80.0	45.8	—

[1] Within ± 1.0% as determined by Kjeldahl analysis.
[2] Described in table 1.
[3] 86.75% casein, 7.82% glycine, 4.26% arginine, and 1.17% methionine.
[4] Cerelose.

TABLE 2

Composition of purified isolated soy diets

	Crude protein,[1] %				
	0	15	30	50	70
	% of diet	% of diet	% of diet	% of diet	% of diet
Protein-free basal [2]	20.0	20.0	20.0	20.0	20.0
Isolated soy protein [3]	—	17.1	34.2	57.0	80.0
Dextrose [4]	80.0	62.9	45.8	23.0	—

[1] Within ± 1.0% as determined by Kjeldahl analysis.
[2] Described in table 1.
[3] 96.5% assay protein (Skidmore Enterprises), 2.5% glycine, and 1.0% methionine.
[4] Cerelose.

ment. The results are presented in table 4. With the exception of the fortuitous value observed on the second day, the size of the pancreas of the chicks fed the 30% protein diet remained fairly constant when expressed as a percentage of the body weight. The pancreas and the total body lost weight throughout the 10 days of protein-free feeding.

The proteolytic activity in the pancreas of the protein-fed chicks remained relatively constant during the 10-day experimental period. In the chicks fed the protein-free diet, the proteolytic activity decreased markedly in 24 hours, but thereafter appeared to increase gradually.

Experiment 2 was performed to determine whether any change in the size of or the protease synthesis by the chicks' pancreas would result from feeding different levels of dietary protein to normal and depleted chicks. One hundred normal chicks were divided into 5 groups of 20 chicks each and were fed diets containing zero, 15, 30, 50 or 70% protein. Five chicks from each treatment were killed after 1, 2, 4 and 7 days of treatment. The same experimental design was followed using 100 depleted chicks after they were fed the protein-free diet for 10 days. The data are summarized in tables 5 and 6.

TABLE 4

Pancreas size and proteolytic activity[1] in chicks fed 0 and 30% soy protein diets (experiment 1)

Dietary protein		Days on treatment				
		1	2	4	7	10
%						
30	Pancrease size, g	1.06	1.44	1.25	1.45	1.47
	Pancreas size, % body wt	0.24 ± 0.02[2]	0.32 ± 0.02	0.23 ± 0.01	0.23 ± 0.03	0.21 ± 0.01
	Body wt, g	447 ± 7	445 ± 5	532 ± 18	616 ± 22	701 ± 19
	Proteolytic activity	13.2 ± 2.3	8.4 ± 2.1	8.0 ± 2.6	10.6 ± 1.3	14.3 ± 3.0
0	Pancreas size, g	0.95	0.87	0.64	0.51	0.45
	Pancreas size, % body wt	0.22 ± 0.02	0.22 ± 0.01	0.18 ± 0.03	0.16 ± 0.02	0.16 ± 0.01
	Body wt, g	434 ± 12	388 ± 15	357 ± 13	317 ± 13	282 ± 7
	Proteolytic activity	1.7 ± 0.2	1.8 ± 0.4	2.9 ± 0.8	3.1 ± 1.1	4.5 ± 1.3

[1] The amount (mg) of lyophilized chick pancreas required to liberate a quantity of tyrosine equal to that liberated by a 10-mg experimental sample.
[2] Each value is the mean of observations in 5 chicks ± SE.

TABLE 5

Proteolytic activity[1] of normal and depleted chicks fed different levels of dietary protein (exp. 2)

Pre-treatment nutritional state[2]	Treatment	Dietary protein	Proteolytic activity,[1] days on treatment			
			1	2	4	7
		%				
Normal	1	0	3.9 ± 0.2[3]	3.7 ± 0.5	5.9 ± 1.0	4.3 ± 0.9
	2	15	5.0 ± 0.9	6.9 ± 1.3	9.1 ± 1.3	5.3 ± 1.7
	3	30	8.8 ± 1.3	11.6 ± 1.7	10.5 ± 3.0	13.0 ± 4.5
	4	50	15.4 ± 1.2	11.1 ± 2.0	12.1 ± 2.4	14.2 ± 1.2
	5	70	12.1 ± 0.6	11.7 ± 1.9	13.7 ± 3.6	21.7 ± 4.3
Depleted	6	0	6.8 ± 0.4	8.0 ± 1.0	5.5 ± 1.0	5.0 ± 0.5
	7	15	11.6 ± 4.1	14.1 ± 3.9	10.6 ± 1.8	12.3 ± 1.6
	8	30	18.0 ± 1.2	12.1 ± 2.5	18.0 ± 5.4	32.7 ± 1.8
	9	50	13.8 ± 1.4	25.5 ± 2.8	14.5 ± 4.7	11.4 ± 0.9
	10	70	21.3 ± 2.2	20.5 ± 1.1	9.7 ± 0.9	11.1 ± 1.5

[1] The amount (mg) of lyophilized chick pancreas required to liberate a quantity of tyrosine equal to that liberated by a 10-mg experimental sample.
[2] Normal chicks received a 30% soy protein diet prior to treatment. Depleted chicks received a protein-free diet for 10 days prior to treatment.
[3] Each value is the mean of observations in 5 chicks ± SE.

TABLE 6

Pancreas size of normal and depleted chicks fed different levels of dietary protein (exp. 2)

Pre-treatment nutritional state [2]	Treatment	Dietary protein	Pancreas size, days on treatment							
			1		2		4		7	
		%	*g*	*% body wt*	*g*	*% body wt*	*g*	*% body wt*	*g*	*% body wt*
Normal	1	0	0.79	0.26 ± 0.01 [3]	0.72	0.24 ± 0.07	0.65	0.25 ± 0.07	0.56	0.24 ± 0.03
	2	15	0.72	0.22 ± 0.01	0.99	0.28 ± 0.02	1.06	0.28 ± 0.01	1.01	0.27 ± 0.02
	3	30	0.98	0.28 ± 0.02	1.25	0.32 ± 0.02	1.23	0.31 ± 0.01	1.41	0.29 ± 0.02
	4	50	1.35	0.38 ± 0.02	1.33	0.37 ± 0.03	1.40	0.34 ± 0.02	1.67	0.34 ± 0.02
	5	70	1.12	0.36 ± 0.02	1.30	0.39 ± 0.03	1.58	0.41 ± 0.04	1.60	0.41 ± 0.01
Depleted	6	0	0.51	0.22 ± 0.04	0.54	0.24 ± 0.02	0.55	0.26 ± 0.01	0.51	0.26 ± 0.03
	7	15	0.64	0.24 ± 0.01	1.01	0.36 ± 0.05	1.33	0.39 ± 0.04	1.23	0.29 ± 0.01
	8	30	0.82	0.29 ± 0.01	1.36	0.44 ± 0.03	1.57	0.42 ± 0.04	2.19	0.43 ± 0.05
	9	50	0.96	0.34 ± 0.03	1.41	0.42 ± 0.03	1.87	0.50 ± 0.04	1.93	0.47 ± 0.03
	10	70	0.86	0.32 ± 0.03	1.33	0.45 ± 0.04	1.60	0.47 ± 0.04	1.95	0.49 ± 0.04

[1] Normal chicks received a 30% soy protein diet prior to treatment. Depleted chicks received a protein-free diet for 10 days prior to treatment.
[2] Each value is the mean of observations in 5 chicks ± SE.

After one day of feeding 50 and 70% protein diets to the normal chicks (treatments 4 and 5), there was a slight increase in proteolytic activity. At the same time, there was decreased activity with the 15% protein diet (treatment 2). By the second day, the proteolytic activity in the normal chicks fed the 50 and 70% protein diets had decreased to the value observed in those receiving the 30% protein diet (treatment 3). There was an increase in enzyme activity on the seventh day for those normal chicks in treatment 5 (70% protein).

The pancreas of the depleted chicks increased in protease content within 24 hours after protein feeding. By the second day of treatment, those depleted chicks fed the 50 and 70% protein diets (treatments 9 and 10) had nearly twice the proteolytic activity observed in those chicks fed the 30% protein diet (treatment 5). However, on the fourth and seventh days the proteolytic activity of the depleted chicks fed the 30% protein diet increased, whereas that in the chicks fed the 50 and 70% protein diets decreased. On the seventh day of protein-free feeding (treatment 6), there was proteolytic activity in 10 mg of pancreas equivalent to 5.0 mg of LCP. In essence, this was the value for 17 days of protein-free feeding (10-day depletion period plus 7 days of treatment).

The mean size of the pancreata in experiment 2 is tabulated in table 6. When expressed as a percentage of the body weight, the pancreas size in the normal chicks generally increased with increasing level of protein from 15 to 70% after the first day of treatment. The same trend was evident in the depleted chicks, but the actual size of the pancreas in treatments 7 through 10 after the first day was greater than that of the pancreas of the normal chicks on the corresponding treatments (treatments 2 through 5).

The body weights of the chicks in experiment 2 at the time of killing appear in table 7. The 30 and 50% protein diets afforded the best growth in the normal chicks; and the best growth among the depleted chicks occurred with the 30% protein diet. However, the depleted chicks grew at a much greater rate than the normal chicks. On the first day of treatment, the depleted chicks were smaller than the

normal chicks, but by the seventh day, the weights of the normal and the depleted chicks receiving protein-containing diets were comparable.

A preliminary study showed that the mitotic rate in the pancreas of chicks fed a protein-free diet was less than 1% during the 4-hour colchicine treatment period. Experiment 3 was conducted to determine the mitotic rate in the pancreas of normal and depleted chicks fed 30, 50 or 70% protein diets for 2 and 7 days. Each datum in table 8 is the mean of observations in 3 chicks.

The levels of dietary protein used had no effect on the mitotic rate in normal chicks. In the depleted chicks, a normal rate of cell division had resumed by the second day and had nearly doubled by the seventh day on the 30 and 50% protein diets. The mitotic rate was somewhat less in the depleted chicks fed the 70% protein diet than in the depleted chicks fed the 30 or 50% protein diets for 7 days.

Casein protein was substituted for the isolated soybean protein at the 30 and 70% protein levels in experiment 4. The purpose of this study was to determine whether the effects of casein protein on the pancreas were comparable to those observed with the isolated soybean protein. The measurements of pancreatic weight and proteolytic activity in normal and depleted chicks were made after 4 days of treatment (table 9).

There was little difference in the proteolytic activities observed in either the normal or depleted chicks fed the 30 or 70%

TABLE 7

Body weights at time of killing of normal and depleted chicks fed different levels of dietary protein (exp. 2)

Pre-treatment nutritional state [1]	Treatment	Protein	Body wt, days on treatment			
			1	2	4	7
		%	g	g	g	g
Normal	1	0	301 ± 8 [2]	293 ± 9	255 ± 13	236 ± 13
	2	15	317 ± 20	355 ± 9	385 ± 15	376 ± 18
	3	30	347 ± 11	394 ± 19	405 ± 19	487 ± 14
	4	50	350 ± 16	363 ± 10	413 ± 15	496 ± 22
	5	70	318 ± 12	339 ± 13	388 ± 9	397 ± 35
Depleted	6	0	228 ± 4	245 ± 13	209 ± 5	196 ± 4
	7	15	260 ± 11	276 ± 14	346 ± 6	418 ± 19
	8	30	284 ± 2	313 ± 21	376 ± 18	511 ± 26
	9	50	283 ± 10	337 ± 6	380 ± 15	411 ± 11
	10	70	270 ± 19	295 ± 8	347 ± 20	396 ± 34

[1] Normal chicks received a 30% soy protein diet prior to treatment. Depleted chicks received a protein-free diet for 10 days prior to treatment.
[2] Each value is the mean of observations in 5 chicks ± SE.

TABLE 8

Mitotic rate [1] in the pancreas of normal and depleted chicks fed 30, 50 or 70% soy protein diets for 2 and 7 days (exp. 3)

Pre-treatment nutritional state [2]	Dietary protein	Day 2		Day 7	
		Mitotic rate [1]	Pancreas size	Mitotic rate	Pancreas size
	%	%	g	%	g
Normal	30	11.2 ± 4.3 [3]	1.56 ± 0.14	10.0 ± 0.2	1.75 ± 0.20
	50	13.4 ± 2.4	1.55 ± 0.08	10.2 ± 2.6	1.50 ± 0.11
	70	10.9 ± 0.6	1.22 ± 0.04	10.5 ± 2.3	1.82 ± 0.12
Depleted [4]	30	12.1 ± 3.7	1.46 ± 0.07	18.0 ± 1.2	2.00 ± 0.16
	50	11.8 ± 4.1	1.30 ± 0.15	20.6 ± 2.4	1.70 ± 0.06
	70	8.6 ± 2.2	0.97 ± 0.17	11.4 ± 3.8	1.73 ± 0.25

[1] The percentage of cells entering mitosis during the 4 hours following colchicine injection.
[2] Normal chicks received a 30% soy protein diet prior to treatment. Depleted chicks received a protein-free diet for 10 days prior to treatment.
[3] Each value is the mean of observations in 3 chicks ± SE.
[4] The mitotic rate observed after 10 days of protein-free feeding was less than 1%.

TABLE 9

Pancreas size and proteolytic activity[1] of normal and depleted chicks after they were fed casein diets for 4 days (exp. 4)

Pre-treatment nutritional state[2]		Protein content of diets, %		
		0	30	70
Normal	Pancreas size, g	0.71	1.24	1.07
	Pancreas size, % body wt	0.31 ± 0.03[3]	0.31 ± 0.02	0.30 ± 0.02
	Proteolytic activity	6.9 ± 1.0	13.8 ± 2.5	12.3 ± 1.7
Depleted	Pancreas size, g	0.47	1.07	1.27
	Pancreas size, % body wt	0.25 ± 0.01	0.37 ± 0.02	0.44 ± 0.05
	Proteolytic activity	6.6 ± 1.2	11.4 ± 1.9	14.4 ± 2.2

[1] The amount (mg) of chick pancreatin required to liberate a quantity of tyrosine having an optical density equal to that obtained with a 10-mg experimental sample.
[2] Normal chicks received a 30% soy protein diet prior to treatment. Depleted chicks received a protein-free diet for 10 days prior to treatment.
[3] Each value is the mean of 5 chicks ± SE.

casein protein diets. However, the pancreas of the depleted chicks fed the 70% casein protein diet was larger than that of both the depleted chicks fed the 30% casein protein diet and the normal chicks fed the 70% protein diet. The size of the pancreas expressed as a percentage of the body weight indicates that the pancreas of the depleted chicks increased in size at a greater rate than did the total body. These results suggest that the rapid growh of pancreatic tissue during protein repletion was not caused by any unique property of the isolated soybean protein.

DISCUSSION

Ju and Nasset (10) measured the nitrogen loss in the pancreas of rats fed a nonprotein diet and found that there was a considerable loss of nitrogen during the first 96 hours. Thereafter, the rate of nitrogen loss decreased. In the present experiment, the pancreas of the chicks fed the protein-free diet lost weight at a greater rate than did the total body for the first 4 days of treatment. On both the seventh and tenth days of protein-free feeding, the pancreas weight was 0.16% of the total body weight, indicating a parallel rate of loss in both pancreas and body weight from the seventh to the tenth day of treatment.

A comparison of the pancreas size data in experiment 1 (table 4) with that in experiment 2 (table 6) suggests that age or size of the chicks, or both, may influence the rate of weight loss by the pancreas. In treatment 1 of experiment 2 (table 6), the pancreas size remained relatively constant when expressed as a percentage of the body weight. In experiment 1, this same measurement for the protein-free treatment decreased with time for the first 4 days. The chicks used in experiment 2 were 1 week younger and about 150 g lighter than those used in experiment 1. Part of the weight lost by the pancreas of chicks fed the protein-free diet was probably due to the secretion of digestive enzymes into the gut. However, the fact that these pancreata contained proteases after 17 days of protein-free feeding indicates that there was a continuous loss of nonproteolytic substances. A greater proportion of these substances in the pancreas of the older chicks may have accounted for the more rapid loss in weight by these pancreata. Also, the greater total protein requirement of the larger chicks may have placed a greater drain on the labile protein of their pancreas.

It appears that the amount of proteolytic activity in a 10-mg sample of pancreas from the chicks fed the protein-free diet tended to increase with time. However, since the weight of the pancreas was decreasing during this time, it is likely that the total amount of protease secreted was not increasing. The stimulus and the supply of amino acids must have come mainly from the endogenous secretions of the proventriculus and the small intestine. This endogenous nitrogen source may be considerable even with a protein-free diet (11), but it is unlikely that it would increase with time.

Perhaps the most significant observation in experiment 2 was the marked difference in the response of the normal and the depleted chicks when fed diets varying in protein content. In the normal chicks, the pancreas size generally increased as the level of dietary protein increased after the first day of treatment. The same was true for the depleted chicks, but their pancreas was heavier than that of the normal chicks fed the corresponding diets for the same length of time.

The mitotic rate measurements indicated that the growth of the pancreas of the normal chicks was due primarily to cellular hypertrophy. In the depleted chicks, the pancreatic growth observed after feeding protein-containing diets appeared to result from hyperplasia. These results were similar to those reported by Leduc (7) in her study of the mitotic activity in the liver of starved rats after refeeding with diets varying in protein content. It is felt (12) that during starvation, the cells which would normally divide are held in interphase. When nutriment does become available, an increased number of cells would be ready to divide resulting in a very high number of mitoses. To what extent this mitotic rate would persist can not be ascertained from the present study, but the data suggest that an overgrowth of the pancreas had occurred within 7 days of feeding protein to the depleted chicks.

A comparison between the proteolytic activities observed in normal chicks fed the 15% protein diet and in those fed the 70% protein diet tends to support previous reports which indicate that the amount of proteolytic activity in the pancreas increases when the dietary protein level increases. However, when the proteolytic activities observed in the chicks fed the 30 and 50% protein diets are included in the comparison this statement is not entirely correct. The feeding of a 15% protein diet to normal chicks caused a decrease in the proteolytic activity, whereas the 50 and 70% protein diets had, essentially, no effect on the proteolytic activity. In terms of chick growth and practicability, the 30% protein diet was the best diet of those used. Without knowing which level of protein is optimum for the animal, the conclusion that the amount of protease secreted by the pancreas varies directly with the level of dietary protein should be made with reservation. Based upon the present study, a statement to the effect that with normal chicks surfeit protein feeding results in a greater synthesis of pancreatic protease than does the feeding of an inadequate level of protein is justifiable.

When depleted chicks were fed protein-containing diets, a rapid increase in the synthesis of proteolytic enzymes occurred. The data collected for the first 2 days of treatment indicated that the level of dietary protein did have a direct bearing upon the synthesis of pancreatic proteases. After the first 2 days, the synthesis of proteases in the chicks fed the 50 and 70% protein diets decreased. Comparison of the mitotic rates observed in the pancreas of the depleted chicks fed the 30, 50, and 70% protein diets for 2 and 7 days with the proteolytic activity observed on these days suggested that protease synthesis was least when the mitotic rate was greatest. This observation coincides quite well with the idea referred to by Weiss (13) that a cell which divides does not function, and a cell which functions maximally does not divide.

One of the mechanisms which may be involved in the regulation of cell division is the relationship between cytoplasmic volume and nuclear volume (14). The cytoplasmic mass may become too large to be served by a single nucleus so that division must take place. The results of the present investigation indicate that this may have occurred in the acinar cells of the depleted chicks' pancreas. At the end of the 10-day depletion period these cells were pyknotic and nondividing. However, within 24 hours of protein feeding these cells were synthesizing large amounts of proteases indicating that the metabolism in the cytoplasm had immediately increased and within 48 hours the mitotic rate had been restored to a normal level.

The superior growth rate observed in the depleted chicks when allowed protein-containing diets parallels the observations reported by Jennings and Morris (15). These authors reported that, in pullets, a period of low protein feeding resulted in greater-than-normal productivity when op-

timal levels of protein were refed. The ability of the depleted chicks' pancreas to synthesize increased amounts of proteases in a very short time, undoubtedly, played a major role in the ability of these chicks to regain their lost body weight and to exhibit a greater growth rate than was observed in normal chicks.

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Tryptophan Utilization in a Threonine-induced Amino Acid Imbalance in Weanling Rats: Plasma amino acid and liver pyridine nucleotide concentrations [1]

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ABSTRACT Growth studies with niacin-free, low casein diets, to which threonine had been added, indicated that this amino acid-imbalanced mixture could be well-utilized when the protein was fed separately but not when the entire diet was fed ad libitum. In the present experiment 4 treatments were used involving both ad libitum and separate feeding of a balanced protein (casein) and an imbalanced protein mixture (casein with added threonine). Poor growth occurred whenever the imbalanced protein was fed ad libitum, regardless of pre-treatment of the animal. Since a growth depression resulted when animals were fed the imbalanced diet ad libitum following a week of good growth with the same protein mixture fed separately, there appeared to be no adaptation to the imbalanced mixture per se. The differences in plasma amino acid patterns did not explain the differences in the growth and acceptability of the imbalanced protein by the 2 feeding methods. Plasma threonine was elevated in animals fed the imbalanced diet regardless of the growth of the animals. Plasma tryptophan did not appear to be depressed more than other amino acids, and the lowest plasma amino acids were observed with the group fed the imbalanced mixture separately in which good growth had occurred. No differences in liver pyridine nucleotide concentrations were observed between treatments at either of the 2 time-intervals tested to suggest an alteration in the tryptophan-niacin pathway. It appeared that use of a feeding method which ensured ingestion of larger quantities of protein per feeding may have improved the utilization of amino acids from an imbalanced mixture.

In studies on tryptophan-niacin relationships in adult human subjects, Goldsmith et al. (1) observed that with diets low in tryptophan and niacin, symptoms of niacin deficiency developed more rapidly in subjects fed corn diets than wheat diets, although the levels of tryptophan and niacin were equivalent. Since the effect did not appear to be due to niacin per se (2), it was suggested that an amino acid imbalance may have caused a difference in tryptophan utilization. Most studies on tryptophan utilization in amino acid imbalance have been made with experimental animals under ad libitum feeding conditions and when the percentage of either tryptophan or niacin in the diet was increased, a concomitant increase in total food consumption occurred (3). Thus, the relative effects of tryptophan or niacin per se, in correcting the imbalance, were difficult to evaluate.

Several amino acid imbalances involving different proteins and limiting amino acids have been characterized by a depression in both food intake and growth, and appear to have been associated with an increased requirement for the limiting amino acid (4). Of these combinations only the imbalance which involves tryptophan is further complicated by the interrelationship with a vitamin, that is, niacin. Studies have indicated, however, that imbalanced mixtures can be used as efficiently as balanced mixtures if equal quantities are consumed. Equalization of food intake has been obtained by increasing the consumption of the imbalanced protein by insulin treatment (5), cold environment (6), previous protein depletion (7) or by an alteration in the method of feeding (8).

In an attempt to regulate the protein intake of weanling rats fed an amino acid imbalanced diet involving tryptophan, Morrison and Caldwell (8), using a low

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casein, niacin-free diet containing threonine, controlled the protein consumption (and thus the tryptophan intake) by the use of a separate-feeding method. Measured amounts of the protein portion of the diet were fed separately, with the remainder of the diet allowed ad libitum. In these studies, using a wide range of protein intakes, although a growth depression always resulted when all the components of the imbalanced diet were fed ad libitum, the growth depression was eliminated by use of the separate-feeding method. The addition of either niacin or tryptophan to the diets of animals fed ad libitum also prevented the growth depression.

Since no difference in growth of weanling rats was observed when equal quantities of the balanced protein (casein) were given by the 2 feeding methods, the reason for the difference in response with the imbalanced diet was not clear. One possibility existed that, by using this separate-feeding procedure, animals adapted to the imbalanced mixture during the experimental period, and were thus able to use it well; a further possibility was that these animals actually utilized it in a different manner from the same protein mixture fed ad libiutm. If the latter were true, results of experiments with imbalanced proteins in which various measurements were made after a test protein had been consumed during a short period might not be applicable to a situation where the diet had been offered ad libitum, and ingested over a long period of time.

The experiments reported here were designed to study the possibility that the animals could adapt to the threonine-induced amino acid imbalance, and thus utilize this mixture as efficiently as a balanced mixture. Liver pyridine nucleotides were measured as an indication of the extent to which growth depression might have been related to the tryptophan-niacin interrelationship. Changes in plasma amino acids were determined to learn whether there were any differences between treatments which might be related to the growth depression or food consumption.

EXPERIMENTAL

Male weanling rats of the Holtzman strain weighing 45 to 55 g were maintained with a 10% casein, niacin-free diet for 2 to 3 days before experiments were begun. Animals were fed by 2 methods: 1) the entire diet was allowed ad libitum, and 2) the protein portion of the diet was fed separately twice daily at 8 AM and 5 PM, with the other components combined in a protein-free diet and fed ad libitum. The animals consumed the protein within a 10- to 15-minute period. Prior to the experimental period, animals were trained for 2 to 3 days to eat protein separately.

The protein-free basal diet had the following percentage composition: salts B (9), 5.0; niacin-free vitamin mix in sucrose (9), 0.25; choline chloride, 0.15; fat-soluble vitamins in corn oil, 5.0; cellulose, 2.0; and sucrose, 87.6. The fat-soluble vitamin mixture provided 400 IU vitamin A, 200 IU vitamin D and 10.0 mg α-tocopherol/100 g of diet.

Two protein sources, a balanced mixture of vitamin-free casein supplemented with DL-methionine, referred to as Cas and an imbalanced mixture referred to as Cas-threonine, were used in all experiments. The proportions used in the 2 protein mixtures were similar to those used previously (8) and were as follows:

Cas (Cas):	
Vitamin-free casein	8.0
DL-methionine	0.3
Total	8.3
Cas-threonine (Cas-Thr):	
Vitamin-free casein	8.0
DL-Methionine	0.30
DL-Threonine	0.36
L-Lysine·HCl	0.162
DL-Phenylalanine	0.140
L-Histidine·HCl	0.038
Total	9.000

Animals fed ad libitum received either 8.3% of the Cas diet or 9.0% of the Cas-threonine diet, so that the percentage of vitamin-free casein (and therefore tryptophan) in the diets of both groups was equal. The protein mixture in each case replaced an equal amount of sucrose in the protein-free basal diet. Animals fed protein separately received 0.83 g Cas/day

(the amount of protein consumed by weanling rats fed ad libitum an 8.3% Cas diet in a preliminary experiment) or 0.90 g Cas-threonine/day in 2 equal portions at 8 AM and 5 PM. The actual amount of vitamin-free casein and thus tryptophan in the 2 diets was equal. In the final experiment, L-threonine replaced DL-threonine and the proportion of this amino acid in the imbalancing mixture was reduced from 0.36 to 0.18. Animals fed protein separately received 0.88 g Cas-L-threonine per day; and animals fed ad libitum received an 8.8% Cas-L-threonine diet.

In all experiments, 4 treatments were used so that balanced and imbalanced protein mixtures could be fed both ad libitum and by the separate-feeding method. The treatments were designated as Cas-Separate, Cas-threonine-Separate, Cas-Ad libitum and Cas-threonine-Ad libitum. Except where otherwise noted, 5 rats were used for each treatment and the mean weight between groups did not vary by more than one gram. Individual weight gains and food consumption were measured 3 times weekly during the experimental period.

In the final experiment, after a 2-week growth period using diet and procedures above, animals were killed at 2 time-intervals, 4 and 24 hours, following a protein feeding. Previous studies [2] had indicated that when the daily allotment of protein (0.8 to 0.9 g) was fed once, twice or three times daily at 8 A.M., 8 A.M. and 5 P.M., or 8 A.M., 12:30 P.M. and 5:00 P.M., the imbalanced protein mixture was well-accepted and utilized. Therefore, in this study, blood amino acids and liver pyridine nucleotides were measured at times which had been established previously as intervals between feedings which allowed good utilization of an imbalanced mixture.

In the groups fed protein separately, the protein-free diet was left in the cages until the animals were killed. For animals fed ad libitum, a protein-free diet was substituted for the total diet either 4 or 24 hours before the animals were killed, but neither the actual time nor the amount of the last protein meal could be determined in these animals.

Animals were anesthetized with ether, and a heparinized syringe was used to take 2 to 4 ml of blood by heart puncture for amino acid analysis. Plasma from each rat was prepared and analyzed microbiologically for free tryptophan with *Leuconostoc mesenteroides* by the method of McLaughlan et al. (10). The remaining plasma was pooled by experimental groups and the pattern of free amino acids determined using a Technicon automatic analyzer by the method of Hamilton (11). Livers were removed immediately after taking the blood sample. Oxidized pyridine nucleotides were determined in the liver by the method of Feigelson et al. (12).

RESULTS

Rate of weight gain with different methods of feeding balanced and imbalanced protein mixtures

Male weanling rats, trained to eat protein separately, were divided into 4 groups of 5 rats each. Half of the animals received either Cas (balanced mixture) or Cas-threonine (imbalanced mixture) incorporated into the basal diet and fed ad libitum; the remaining animals were fed the same protein mixtures separately, twice daily, with protein-free diet available ad libitum. The experiment was repeated and the results are shown in figure 1.

The animals fed the Cas-threonine ad libitum grew more slowly than all other animals throughout the entire 2-week period, and the low growth rate was associated with low food consumption. When Cas-threonine was fed separately, the growth rate was as great as with Cas fed by either procedure and food consumption was not depressed. Results of studies with other imbalanced diets (13) showing an initial decrease in food consumption and subsequent increase of intake, had suggested that animals could adapt to certain imbalanced diets after a few days. The present results showed no adaptation to the ad libitum feeding of Cas-threonine diet during the 2-week experimental period, and no increase in rate of growth.

[2] Marshall, M. 1962 The effect of sucrose and cornstarch on protein utilization in rats with different feeding patterns, M.S. Thesis, Cornell University.

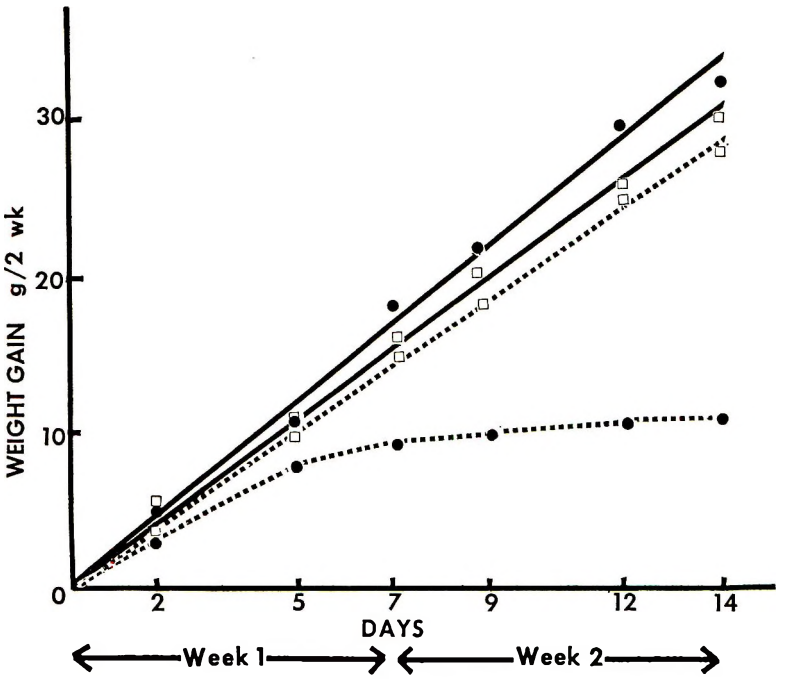

Fig. 1 Rate of weight gain by rats fed Cas or Cas-threonine ad libitum and by a separate-feeding method. Cas-Separate □ —— □, Cas-threonine-Separate ● —— ●, Cas-Ad libitum □ – – – □, Cas-threonine-Ad libitum ● – – – ●.

While the method of feeding may have influenced the growth rate of rats fed the imbalanced protein, no effect of feeding method was observed with the balanced protein.

Effect of change in feeding method on the growth response to balanced and imbalanced protein mixtures

The animals always appeared to utilize the imbalanced mixture well when it was fed separately. If some adaptation to the imbalanced mixture occurred, the animals should have continued to utilize this mixture when the method of feeding was changed from separate to ad libitum during the experimental period. Weanling rats, previously trained to eat protein separately, were put into 4 groups and during week 1 of the experimental period received one of the 4 treatments described previously (Cas-Separate, Cas-threonine-Separate, Cas-Ad libitum, Cas-threonine-Ad libitum). At the end of the first week, each group of animals was divided into 3 equal sub-groups. One of these sub-groups was continued on the same treatment as in week 1; treatments of the other sub-groups for the second week were altered as shown in table 1. Weight gain and food consumption of animals receiving the 4 different treatments during each week of the 2-week experimental period are shown in table 1. At the end of week 1, weight gains were similar for animals fed separately either Cas or Cas-threonine. As observed in the previous experiment, animals fed Cas-threonine ad libitum gained the least weight. Regardless of treatment in week 1, the weight gains of animals fed the imbalanced diet ad libitum during the second week were low.

Since the rate of weight gain may be a better indication of possible adaptation to the imbalanced diet than total gain, growth rates of animals receiving the Cas-threonine imbalanced mixture, by the 2 feeding methods, during weeks 1 and 2 are plotted in figure 2 (average of 2 experiments). Previous to the experimental period, the animals had received a 10% casein diet. The lower rate of gain by the

TABLE 1

Effect of changing feeding method during the experimental period on weight gain and food consumption of rats fed Cas [1] or Cas-threonine [2]

Week 1				Week 2			
Treatment	Wt gain	Food consumption		Treatment	Wt gain	Food consumption	
		Casein [3]	Protein-free			Casein [3]	Protein-free
	g/rat/week	*g/rat/week*			*g/rat/week*	*g/rat/week*	
Cas-Sep [4]	17.9 ± 1.2 [5]	5.6	46	Cas-Sep	12.9 ± 1.2 [5]	5.6	57
				Cas-Ad lib	12.6 ± 1.8	5.6	64
				Cas-Thr Ad lib	7.1 ± 1.8	4.2	50
Cas-Thr Sep	18.9 ± 0.5	5.6	44	Cas-Thr-Sep	15.1 ± 1.0	5.6	54
				Cas-Ad lib	9.2 ± 1.6	4.7	54
				Cas-Thr-Ad lib	2.1 ± 1.7	3.9	44
Cas-Ad lib [6]	13.7 ± 0.8	4.6	50	Cas-Thr-Sep	16.9 ± 1.5	5.6	54
				Cas-Ad lib	13.4 ± 1.5	5.3	61
				Cas-Thr-Ad lib	6.5 ± 1.6	4.0	46
Cas-Thr Ad lib	8.4 ± 0.7	3.5	40	Cas-Thr-Sep	12.3 ± 1.5	5.6	41
				Cas-Ad lib	7.9 ± 1.6	4.0	46
				Cas-Thr-Ad lib	3.3 ± 1.9	3.3	38

[1] Cas contained vitamin-free casein, 8.0; and DL-methionine, 0.3.
[2] Cas-threonine (Cas-Thr) contained vitamin-free casein, 8.0; DL-methionine, 0.30; DL-threonine, 0.36; DL-phenylalanine, 0.140; L-lysine·HCl, 0.162; and L-histidine·HCl, 0.038.
[3] Vitamin-free casein.
[4] Separate-feeding method.
[5] Mean ± SEM; 30 rats/group in week 1; 10 rats/group in week 2.
[6] Ad libitum-feeding method.

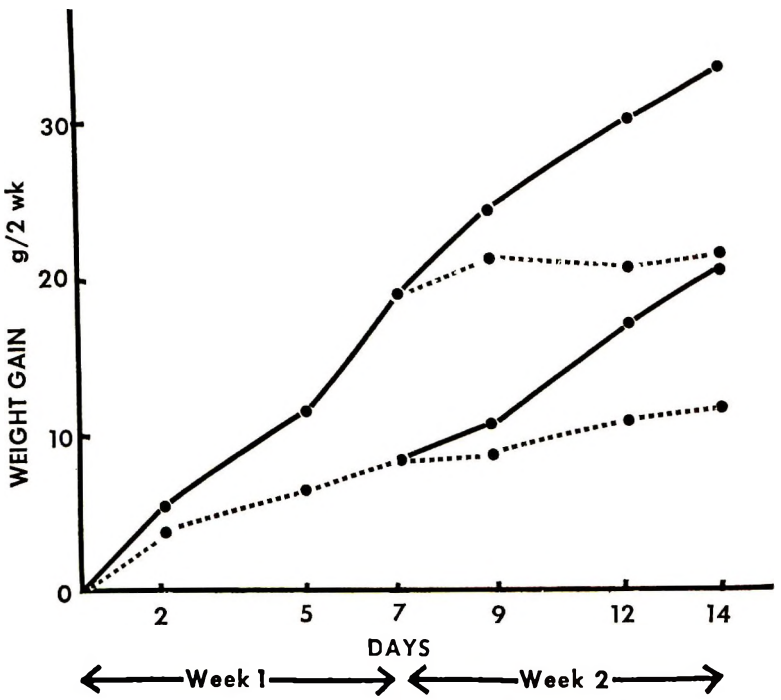

Fig. 2 Effect of changing feeding method on utilization of an imbalanced protein mixture. Cas-threonine-Separate ● —— ●, Cas-threonine-Ad libitum ● – – – ●.

animals fed the imbalanced diet ad libitum was evident within 2 days of the start of the treatment in both week 1 and week 2; food consumption also decreased. In these studies, animals that had apparently utilized the imbalanced protein well for one week (Cas-threonine-Separate) did not maintain the same growth rate when the feeding method was changed. Conversely, animals that had received Cas-threonine-Ad libitum during week 1 and had grown poorly, were immediately able to utilize this imbalanced mixture when the protein was fed separately. In each case, the growth response to a change in treatment was quite rapid. At no time did the animals fed the imbalanced diet ad libitum grow well, but in every case, when the protein was offered separately, it was well-accepted and utilized. Results of this experiment suggested that the difference in acceptability of the imbalanced diet and the growth response with 2 feeding methods could not be explained by the age of the animal, that is, one or 2 weeks post-weaning, previous dietary treatment or possible adaptation to the imbalanced mixture, per se.

Effect of balanced and imbalanced protein mixtures on plasma amino acid and liver pyridine nucleotide concentrations

In groups fed protein separately, blood and liver samples were taken 4 or 24 hours after a protein meal (0.42 g Cas or 0.45 g Cas-threonine). In groups fed ad libitum, blood was taken 4 or 24 hours after removal of the complete diet. All animals were allowed ad libitum access to the protein-free basal diet until they were killed, to minimize tissue breakdown and the possible alteration of plasma amino acid pattern or pyridine nucleotide concentration.

In this experiment, 6 groups of 10 animals each were used. Four groups received the same treatments as in previous experiments: Cas-Separate, Cas-threonine-Separate, Cas-Ad libitum and Cas-threonine-Ad libitum. Two additional treatments in which DL-threonine was replaced by L-threonine in the imbalanced mixture with both feeding methods were designated as Cas-L-threonine-Ad libitum and Cas-L-threonine-Separate.

L- *and* DL-*threonine.* The presence in the imbalanced mixture, of D-threonine (which is not used by the rat) might have complicated interpretation of plasma results, since the analytical procedure used in this study did not distinguish between D- and L-forms of amino acids. Growth response with L- and DL-threonine in the imbalanced mixture was comparable with both feeding methods (table 2). Both L- and DL-threonine caused growth depression with ad libitum feeding and neither caused a depression with separate feeding.

TABLE 2

Weight gain and food consumption of rats fed Cas-threonine with either DL- *or* L-*threonine*

Treatment [1]	Wt gain	Food consumption		
		Casein [2]	Protein-free	Tryptophan [3]
	g/rat/2 weeks	*g/rat/2 weeks*		*mg/rat/2 weeks*
	Fed by separate-feeding method			
Cas	30.5 ± 1.2 [4]	11.2	105	124
Cas-threonine	33.5 ± 1.0	11.2	98	124
Cas-L-threonine	32.6 ± 1.7	11.2	97	124
	Fed ad libitum			
Cas	40.5 ± 3.7	11.2	129	124
Cas-threonine	17.4 ± 2.5	7.6	86	84
Cas-L-threonine	13.4 ± 1.7	7.0	79	77

[1] Cas contained vitamin-free casein, 8.0; and DL-methionine, 0.3. Cas-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; DL-threonine, 0.36; DL-phenylalanine, 0.140; L-lysine·HCl, 0.162 and L-histidine·HCl, 0.038. Cas-L-threonine was the same as Cas-threonine but contained L-threonine, 0.18 in place of DL-threonine, 0.36.

[2] Vitamin-free casein.

[3] Tryptophan content of Cas was 10.67 mg/g. Vitamin-free casein contained 1.3 g tryptophan/16 g N (14), 14.57% N (dry basis) and 6% moisture.

[4] Mean ± SEM; 10 rats/group.

Although plasma amino acids and liver pyridine nucleotides were determined on animals receiving both L- and DL-threonine in the imbalanced mixture, only the results with L-threonine have been reported.

Plasma amino acids. To facilitate comparison between treatments, the concentration of each amino acid has been related to the molar value in the plasma 24 hours after feeding Cas separately (table 3). For reference, the actual value of each amino acid in micromoles per 100 ml plasma at 24 hours, for the group receiving Cas separately, has also been given in table 3.

Separate-fed animals. Four hours after animals were fed protein separately, plasma concentration of each essential amino acid (EAA) with the exception of threonine, was lower with Cas-L-threonine than with Cas; the total of the non-essential amino acids was also lower in the plasma of these animals. The level of tryptophan, the limiting amino acid, was depressed after feeding Cas-L-threonine, but the depression was no greater than that of other EAA's which had not been added to the diet (valine, leucine, isoleucine and arginine). Four hours after feeding protein, rats fed Cas-L-threonine had plasma threonine concentrations which were 3 times higher than those of rats fed Cas, but the elevated plasma threonine had no adverse effect on the growth of these animals.

Twenty-four hours after feeding Cas-L-threonine, plasma threonine levels were still 3 times higher than after feeding Cas alone. Other EAA's were somewhat higher with Cas-L-threonine than with Cas. With the separate-feeding method, although differences existed in plasma amino acid levels with the 2 protein mixtures, growth with the 2 diets was similar.

Ad libitum-fed animals. Since animals fed Cas and Cas-L-threonine ad libitum did not consume equal quantities of diet and neither the time nor amount of the last protein meal was established, plasma amino acid differences might have been anticipated. Although after 4 hours, plasma amino acids might reflect the effect of a protein feeding in separately fed animals, such an interpretation might not be valid for animals fed ad libitum. Of the 2 intervals chosen following ad libitum feeding, the 4-hour measurement may have been influenced by the previous protein meal, but the 24-hour measurement should not have been influenced by the time and amount of the previous protein meal.

After 4 hours, with the exception of threonine, there appeared to be no differences in plasma amino acid concentration which might explain why animals consumed less of the imbalanced mixture (table 3). Plasma threonine was elevated in animals which had received Cas-L-threonine, but the elevation was less with ad libitum feeding than with separate feeding. Although plasma tryptophan appeared to be elevated with Cas-L-threonine, tryptophan concentrations were always low (2.8–10.3 μg/ml plasma) and the variation within each group was large (table 4). After 24 hours, plasma threonine was still elevated in animals originally given Cas-L-threonine, but there were no large changes in other plasma amino acids, including tryptophan, from animals fed Cas (table 3).

In this study, differences in plasma amino acid concentration between balanced and imbalanced proteins fed ad libitum were actually less than differences produced by feeding the imbalanced protein by 2 methods. Although plasma responses varied between ad libitum- and separate-fed rats, the differences did not appear to offer explanations for either the growth depression or refusal of food.

Liver pyridine nucleotides. Liver oxidized pyridine nucleotides (PN) were measured at the same time intervals after feeding protein as amino acids and the results are given in table 4. After 4 hours, there were no significant differences in liver PN concentrations regardless of the protein mixture fed or the method of feeding. After 24 hours, PN concentrations were higher with all treatments than at 4 hours, but again, concentration was not related to weight gain. Although the group receiving Cas-L-threonine ad libitum grew much less than the other 3 groups, PN concentrations of all groups were essentially the same at either time-interval considered.

TABLE 3

Effect of feeding Cas [1] and Cas-L-threonine [2] both separately and ad libitum on the concentration of free amino acids in blood plasma of rats killed 4 and 24 hours following protein feeding

Treatment	Hours after feeding	Thr	Met	Lys	Phe	His	Val	Leu	Ileu	Arg	Try [3]	Total EAA (+ Thr)	Total EAA (− Thr)	Total NEAA
												mg of amino acid/100 ml plasma		
						Plasma score [4]								
						Fed by separate-feeding method								
Cas	24	(9.6)	(2.9)	(34.1)	(5.4)	(6.9)	(10.8)	(9.0)	(5.4)	(12.0)	(1.6)			
	24	100	100	100	100	100	100	100	100	100	100	10.9	9.8	21.5
	4	183	652	147	128	152	182	156	144	94	156	19.1	17.0	32.0
Cas-L-threonine	24	341	110	103	128	122	125	119	131	94	219	15.3	11.4	24.8
	4	743	455	97	128	120	105	59	67	55	87	19.2	10.7	24.8
						Fed ad libitum								
Cas	24	153	121	161	104	117	107	119	115	134	150	15.3	13.5	25.3
	4	115	165	195	137	138	157	140	154	95	88	18.0	16.7	25.5
Cas-L-threonine	24	244	196	124	154	98	116	112	115	87	181	15.4	12.6	23.4
	4	460	152	154	115	120	158	142	150	75	319	20.4	15.1	25.1

[1] Cas contained vitamin-free casein, 8.0; and DL-methionine, 0.3.

[2] Cas-L-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; L-threonine, 0.18; L-lysine·HCl, 0.162; DL-phenylalanine, 0.14; and L-histidine·HCl, 0.038.

[3] Individual samples. Analyzed microbiologically.

[4] Plasma score for each amino acid was calculated as the ratio of micromoles of the amino acid in the test group to micromoles of the amino acid in the group fed Cas separately at 24 hours × 100. Actual micromolar values for each amino acid 24 hours after feeding Cas separately are shown in parentheses. Pooled blood samples; 5 rats/group.

TABLE 4

Plasma tryptophan and liver pyridine nucleotide (PN) concentration in rats fed either Cas [1] or Cas-L-threonine [2]

Treatment	Wt gain	Plasma tryptophan		Liver oxidized pyridine nucleotides			
		4 hr [3]	24 hr [3]	4 hr	24 hr	4 hr	24 hr
	g/rat	*μg/ml plasma*	*μg/ml plasma*	*μg PN/ g liver*	*μg PN/ g liver*	*μg PN/ total liver*	*μg PN/ total liver*
			Fed by separate feeding method				
Cas	30.5 ± 1.2 [4]	5.1 ± 2.5	3.3 ± 1.0	633 ± 34	809 ± 44	2621	2783
Cas-L-threonine	32.6 ± 1.7	2.9 ± 0.1	7.2 ± 2.4	708 ± 61	885 ± 44	3108	3283
			Fed ad libitum				
Cas	40.5 ± 3.7	2.8 ± 1.3	4.9 ± 1.8	609 ± 48	741 ± 25	3313	2682
Cas-L-threonine	13.4 ± 1.7	10.3 ± 1.1	5.8 ± 1.3	647 ± 96	861 ± 60	2297	2669

[1] Cas contained vitamin-free casein, 8.0; and DL-methionine, 0.3.
[2] Cas-L-threonine contained vitamin-free casein, 8.0; DL-methionine, 0.3; L-threonine, 0.18; L-lysine·HCl, 0.162; DL-phenylalanine, 0.14; and L-histidine·HCl, 0.038.
[3] Separate-fed animals killed 4 or 24 hours after protein feeding. Ad libitum-fed animals killed 4 or 24 hours after substitution of protein-free diet for complete diet.
[4] Mean ± SEM; 5 rats/group.

Total oxidized PN's after 4 hours were lowest in the group which received Cas-L-threonine ad libitum and grew poorly, but the livers of these animals were the smallest, and they had consumed the least amount of protein and tryptophan during the experimental period. However, after 24 hours, total liver PN's in animals that had received Cas-L-threonine ad libitum were similar to liver PN's of animals fed Cas by either method.

At the 2 time-intervals studied, although differences were observed in both concentration and total oxidized PN in the liver, the differences did not appear to be related either to growh or to plasma tryptophan level.

DISCUSSION

With weanling rats fed ad libitum the addition of threonine to a low casein, niacin-free diet caused a growth depression with an apparent increased requirement for tryptophan (8). The growth depression was prevented by adding a small amount of tryptophan to the diet of animals fed ad libitum or by feeding the imbalanced protein mixture separately with the remaining dietary components available ad libitum. The reason for the depression in food intake and growth of animals fed the imbalanced diet ad libitum is not clear, nor is the reason clear for the good growth of animals fed the same protein separately.

In the present study, the ability of the animal to utilize the imbalanced diet following changes in the feeding procedure was studied. When the animals were fed the imbalanced mixture separately for one week they grew well, but a change to an ad libitum feeding method produced a depression in food intake and growth. In the reverse situation, with animals fed the imbalanced mixture ad libitum for one week during which poor growth occurred, a change to separate feeding of the same protein mixture resulted in increased growth rate. Since a fairly rapid change in response occurred when the method of feeding the imbalanced protein was altered, it appeared that the animals did not adapt to the imbalanced mixture per se, but perhaps utilized the protein mixture which was fed all at one time differently from the same protein mixture fed ad

libitum. Since the amount of tryptophan required to prevent a growth depression is small, the amount of imbalanced protein fed at one time may have influenced the results. In a previous study (8) the amount of protein available to the animal at any one time was varied using both feeding procedures. When the amount of protein fed separately was varied (0.4 to 0.9 g/rat/day) and distributed in 2 daily feedings, both growth and carcass nitrogen were as high with the imbalanced mixture as the balanced mixture. In ad libitum-fed rats, to increase the amount ingested per feeding, the percentage of the imbalanced mixture (Cas-threonine) was increased from 9 to 13% but growth was lower than that in animals receiving equivalent Cas diets (8-12%). It is unlikely that the quantity of protein consumed per feeding in any of the ad libitum studies was reproduced in the separate-feeding study. With the separate-feeding procedure, no difference in response between balanced and imbalanced protein was noted at any level of intake studied.

Although increasing the quantity of protein consumed per feeding appeared to improve the utilization of Cas-threonine in these studies, reasons for the inability of the rat to utilize small quantities of the same protein mixture under ad libitum feeding conditions are not clear. Since plasma amino acid concentrations have been used to study limiting amino acids in other imbalance situations (15, 16), measurements were made to determine whether there were plasma differences which might explain the different response of the imbalanced diet with the 2 feeding methods. Harper (4) proposed that an amino acid-imbalanced diet stimulates more rapid removal of amino acids from plasma than does a balanced diet. As a consequence of a disproportionately rapid removal of the limiting amino acid from the plasma, an unfavorable ratio of the limiting to other amino acids is produced, and subsequent food intake is depressed.

In the experiment reported here, the plasma amino acid concentrations of the group fed the imbalanced mixture separately were lower than those of any other group. The amino acids which appeared to have been most rapidly removed 4 hours after feeding the protein were valine, leucine, isoleucine, arginine and tryptophan. These amino acids were not added to the protein mixture, and thus their concentration might be lowered most markedly if there was a general increase in uptake of amino acids from plasma. The limiting amino acid, tryptophan, was low in the plasma in all cases, and was not depressed more than other amino acids mentioned. In the groups fed ad libitum, in which the time and amount of the last protein intake could not be established, there appeared to be no difference in the rate of removal of the amino acids from the plasma, between animals fed the balanced and imbalanced mixtures.

Thus in these studies, the more rapid removal of the amino acids from the blood of animals fed the imbalanced mixture separately was not associated with a depressed food intake. Direct comparisons with the work of Harper and co-workers (15, 16) cannot be made, since in the present study, blood was sampled at only 2 time-intervals and the imbalance involved a niacin-free diet. Furthermore, the results did not indicate that the higher level of threonine in the blood of animals fed the imbalanced diet was the factor directly causing the depression in food intake in ad libitum-fed animals, since high levels of threonine were also noted in the separate-fed animals which readily consumed the diet.

Since the addition of niacin to an imbalanced diet fed ad libitum improved food consumption and growth (8), the relative amount of niacin synthesized from tryptophan by the animals fed the imbalanced niacin-free diet by the 2 methods may have been important. If tryptophan was used for synthesis of nicotinamide adenine dinucleotide (NAD), then presumbaly less tryptophan would be available for protein synthesis and growth would be depressed. However, the imbalanced diet fed separately actually produced slightly greater growth than the balanced diet fed by either method. In the present experiment, at the times studied, there was neither a difference in liver NAD of animals fed the balanced or imbalanced diets separately, nor was the concentration of liver NAD in animals fed the imbalanced diet ad libitum

lower than in other groups. However, a single measurement of NAD in the liver would give only the concentration at one particular time and would give no indication of the relative rates of synthesis or breakdown of NAD under each of the experimental feeding conditions.

In the conversion of tryptophan to NAD, tryptophan pyrrolase activity is increased by the presence of tryptophan (17) and is inhibited by NAD, but not by niacin (18). In the present studies with niacin-free, low tryptophan diets, an increase in the quantity of tryptophan ingested at any one time, with a more rapid uptake into the liver could produce an increase in tryptophan pyrrolase activity and a subsequent increase in NAD. This elevated NAD could in turn be inhibitory to tryptophan pyrrolase and more tryptophan would be available for protein synthesis. Previous work in this laboratory[3] has shown that the concentration of NAD increased rapidly in response to a protein meal and decreased again to pre-experimental levels in 7 to 12 hours depending on source and amount of tryptophan. The 2 methods of feeding protein used in this study could provide for different quantities of NAD to be synthesized at one time, by providing different amounts of tryptophan at each meal. A rapid increase in NAD after a large protein meal could inhibit further breakdown of tryptophan, while providing an adequate source of niacin, but if the amount of protein in the meal was low, the stimulatory and inhibitory effects on tryptophan pyrrolase might not be significant.

Differences in the size of a protein feeding at any one time may have been one of the reasons for the apparent contradiction in the utilization of tryptophan in two imbalance situations (19, 20). In these studies weanling rats had been fed ad libitum, niacin-free, low tryptophan diets in which the amino acid imbalance had been caused by the addition of threonine. Although growth depression had occurred in both studies, the results of isotopic studies on the utilization of labeled tryptophan fed on the last day of the experiment were opposing. When animals consumed the labeled tryptophan on the last day of the experiment in a diet fed ad libitum (19) (presumably consuming a small amount of diet at any one time), a higher percentage of the isotope was lost in the urine of the animals fed the imbalanced diet than from controls. When the tryptophan (20) was given with the protein by stomach tube on the last day, thus changing the method of feeding and increasing the quantity of tryptophan and protein consumed at one time, no difference in tryptophan excretion or catabolism was observed between animals fed imbalanced or balanced diets.

In the separate-feeding method, it appeared that adequate niacin and tryptophan from the imbalanced diet were available for good growth of the animals. In addition to possible effects of NAD on enzyme activity, the relative rate of niacin formation from the breakdown of liver NAD may have affected the use at other sites in the body or the amount eliminated in the urine. The effect, on the tryptophan-niacin interrelationship, of the method of feeding an imbalanced protein mixture in which tryptophan is limiting, cannot be fully evaluated without further information about rates of synthesis, breakdown and excretion of metabolites in the tryptophan-niacin pathway.

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Effect of Methionine Supplementation on Experimental Atherosclerosis in Rabbits [1,2]

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ABSTRACT A study was made to determine the effect of methionine on hypercholesteremia and atherosclerosis. Forty-eight New Zealand white rabbits were divided into 4 dietary groups of 12 rabbits each. Groups 1 and 2 received daily supplements of 0.5 g cholesterol and 0.25 g methionine, respectively. Group 3 received 0.5 g cholesterol and 0.25 g methionine daily. Group 4, the control group, received no supplement. Two animals from each group were killed and autopsied after 7, 21, 42, 63, 84, and 105 days of supplementation. Aortic tissue was examined both grossly and histologically for signs of atherosclerosis, and the cholesterol content of aortas, hearts, livers, kidneys, and serum was determined. At 21 days and at each subsequent autopsy interval, all rabbits fed cholesterol and cholesterol plus methionine demonstrated grossly apparent atherosclerosis. Medial involvement occurred quite early in the formation of the plaque. The addition of cholesterol to the ration had no observable biochemical effect of the kidney or aorta, but did affect the cholesterol level of the serum, liver and heart. Total feed intake was related negatively to liver cholesterol. There was no evidence of an inhibiting effect of methionine. It was observed that atherosclerosis was not, in all cases, accompanied by elevated serum cholesterol levels.

Several investigators have reported partial prevention of hypercholesteremia and atherosclerosis by methionine supplementation. Filios and Mann (1), Nath and Saikia (2), and Seidal et al. (3) demonstrated a partial diminution of atherosclerosis in the rat by methionine supplementation. Yamaguchi (4) and Nikilla and Ollila (5) indicated similar results with chickens, as did Kleinrok (6) in pigeons. Mann (7) reported the prevention of high plasma cholesterol or β-lipoprotein levels in monkeys by methionine supplementation. In contrast, Weitzert and Buddecke (8) demonstrated the acceleration of atherosclerosis by methionine in the hen, dog and rat.

Little information is available as to the effect of methionine supplementation on atherosclerosis in the rabbit. The present study was undertaken to provide additional data and to add to the existing knowledge of the effect of methionine on hypercholesteremia and atherosclerosis.

MATERIALS AND METHODS

Forty-eight white male New Zealand rabbits (aged 7–9 months) were divided at random into 4 groups of 12 rabbits each. One group of twelve received a daily supplement of 0.5 g cholesterol in 10 ml of raw peanut oil mixed with 10 g rabbit chow.[3] A second and third group received, respectively, 0.5 g cholesterol plus 0.25 g methionine and 0.25 g methionine alone. A fourth group, the control group, received no supplement other than the 10 ml of raw peanut oil used as a supplement binder for the other 3 groups. Each rabbit was allowed to consume the allotment of treated feed ad libitum, and additional (untreated) feed was given after the treated feed was consumed.

The 4 groups of 12 rabbits were further divided into 6 groups of 2 rabbits each. These groups corresponded with date of killing. After one week, 2 rabbits from each of the 4 supplementation groups (the first slaughter group) were killed by electric shock. Two weeks later the second slaughter group was killed. In succeeding 21-day periods, the third through sixth groups

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[2] Published with the approval of the Director of the New Hampshire Agricultural Experiment Station as Scientific Contribution no. 394.

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

were killed. The experiment lasted for 15 weeks.

Blood samples were taken from all experimental animals before the experiment started at week zero (table 1). Samples were obtained from all surviving animals before killing two at each period of slaughter.

Before the start of supplementation, and on each killing date, weights were recorded and blood samples taken by cardiac puncture. The serum cholesterol levels were determined by the method of Sperry and Webb (9) as modified by Carr and Drekter (10).

At the time of killing, gross study of the aorta was performed. The heart, kidneys and liver were removed and weighed. Minute samples of the above organs and aorta were fixed in either Zenker's solution or formalin and embedded in paraffin for histological and histochemical analysis. The remainder of the aorta and organs were minced, quick-frozen and lypholized.

The lypholized samples were ground into a fine powder and the cholesterol was extracted with chloroform-methanol (2:1). The cholesterol in a one-gram sample of lypholized, ground tissue was found to be completely extracted by standing for 6 hours in 25 ml of chloroform-methanol. The results were compared with extraction of 12, 16, 20 and 24 hours to yield the total amount of cholesterol.

After extraction, the tissue samples were centrifuged and the clear supernatant was withdrawn. Of this sample, 0.2 ml was withdrawn, the extractant removed by heating the sample in a hot water bath, and the cholesterol determined by the Carr and Drekter (10) method. The amount of cholesterol in 0.2 ml of sample was related to the total amount of extractant (25 ml) to obtain the total cholesterol per gram of lyophilized tissue.

Sections of aorta were cut and stained with hematoxylin and eosin to determine the extent of plaque formation. Additional sections were stained with Van Geison's collagen stain and both Weigert's and Verhoeff's elastin stains. Attempts to apply the Schultz [4] cholesterol test failed.

[4] Schultz, A. Zentralbl. Allg. Path., 35: 314, 1924; and Schultz, A., and D. Lohr. Zentralbl. Allg. Path., 36: 529, 1925.

TABLE 1

Mean serum cholesterol levels by diet and length of treatment

Diet	Mean serum cholesterol levels							
	No. of weeks from start of experiment							
	0	1	3	6	9	12	15	
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	
Control	35.2(11) [1]	33.7(12)	36.4(9)	40.7(8)	45.9(6)	41.8(2)	44.7(2)	
Methionine	36.5(11)	34.5(11)	27.6(10)	34.9(8)	38.9(6)	37.1(4)	42.4(2)	
Cholesterol + methionine	64.5(11)	141.4(12)	330.3(9)	478.0(7)	667.3(6)	783.6(4)	944.2(2)	
Cholesterol	49.1(12)	137.3(12)	229.2(9)	411.7(7)	577.7(5)	991.4(4)	1611.0(2)	
Overall means	46.4(45)	87.8(47)	152.4(37)	227.8(30)	321.8(23)	523.7(14)	660.6(8)	

[1] Numbers in parentheses indicate the number of rabbits.

Standard one-way analyses of covariance were used to assess the importance of possible concomitant variables. Those variables considered were total feed, feed per day, initial weight, weight at killing, and the appropriate organ weight. On the basis of these preliminary analyses, a linear model was formulated for each trait. Analysis of variance was then obtained for each model using the method of least squares (11). For those cases where there were significant treatment effects and no interaction with slaughter groups, Duncan's new multiple range test (12) as modified by Kramer (13) was used to test the differences among treatment means.

RESULTS AND DISCUSSION

The unadjusted means for the serum cholesterol levels are shown in table 1, and those for the 4 tissues — kidney, liver, heart and aorta — are shown in table 2. All rabbits survived to the designated killing date, and were in apparent good health during the course of the experiment. Missing observations in tables 1 and 2 were the result of hemolyzed samples and other laboratory conditions. The variation in serum cholesterol from interval to interval for the same animals is shown in table 1. On an individual rabbit basis, this variation was considerably larger, which indicated that serum cholesterol at killing was probably a poor measure of the true level. For this reason, the linear regression of serum cholesterol on time was computed for each rabbit; and it is the predicted serum cholesterol level at the time of killing which has been analyzed.

Table 3 shows the results of the least squares analysis for each of the 5 traits. The results of the preliminary analyses are indicated by those factors which were included in the analyses of variance. Several

TABLE 2

Mean tissue cholesterol levels by diet and length of treatment

Diet	Mean tissue cholesterol levels						Overall means
	No. of weeks from start of experiment						
	1	3	6	9	12	15	
	mg/g dry tissue		*mg/g dry tissue*		*mg/g dry tissue*		*mg/g dry tissue*
Control							
Kidney	18.2 [1]	13.8	18.2	15.6	20.1	15.9	16.9
Liver	14.6	14.8	18.4	15.7	12.0	12.6	14.7
Heart	4.8	2.1	2.9	1.1	1.9	2.4	2.1
Aorta	15.4 *	13.4	10.3	13.5	10.8	15.1	12.9
Methionine							
Kidney	18.1	17.2	18.9	16.9	16.1	25.3	18.7
Liver	11.1	9.0	19.8	10.4	9.4	17.5	13.4
Heart	3.4 *	1.9	4.2	1.1	2.7	1.8	2.4
Aorta	11.8	18.2	10.8	10.2	10.8	14.8	12.8
Cholesterol + methionine							
Kidney	20.8	16.8	23.3	26.3	23.5	27.0	22.9
Liver	14.6	58.9	74.4	115.4	67.3	86.3	69.5
Heart	0.9	5.7	10.3	13.0	5.0	12.4	7.9
Aorta	13.2	10.3	13.7	20.4	38.6	31.3	21.2
Cholesterol							
Kidney	20.5	21.1	21.4	20.4	16.8	29.0	21.8
Liver	18.9	40.6	65.4	68.6	50.5	179.7	70.6
Heart	3.1 *	5.3	6.1	10.2	6.0	16.6	8.3
Aorta	11.4	20.0	16.9	25.7	34.6	41.4	25.0
Overall means							
Kidney	19.4	17.2	20.4	19.8	19.5	24.3	20.1
Liver	14.8	31.6	44.5	52.5	34.8	74.0	42.0
Heart	2.2	3.7	5.8	6.3	3.9	8.4	5.2
Aorta	12.6	15.8	12.9	17.5	23.7	25.6	18.1

[1] All means have 2 observations except those with an asterisk which have only one. The overall means have the number of observations equal to the total for that row or column.

TABLE 3

Analyses of variance for traits studied

Source of variation	Mean squares							
	df	Kidney	Liver	Serum	df	Heart	df	Aorta
Diet	3	53.29	8,409 *	83,011 *	3	87.33	3	320.00
Slaughter group	5	41.34	1,812 *	20,073 *	5	6.88	5	125.1
Diet × slaughter group	15	14.55	1,033 *	9,328	15	10.52	15	90.0
Regression on feed consumed	1	79.96	3,525 *	49,815 *	1	0.42	1	162.0
Regression on slaughter wt	1	1.55	389	656	1	19.55	1	5.3
Regression on heart wt	—	—	—	—	1	18.29	—	—
Residual	22	35.87	292	5,899	19	7.97	21	126.8
Total	47				45		46	

* Significant at the 5% level.

factors which were initially thought to be important, were of very little importance when all factors were considered simultaneously.

There was no evidence for an effect of treatment on kidney or aorta cholesterol, nor for an effect of any other factor on these traits. However, the data in table 2 appear to show an increase in cholesterol toward the end of the experiment, indicating that the effect, if any, might be delayed.

The data in table 2 indicate an immediate effect of treatment on liver cholesterol. The analysis of variance substantiates this observation, and shows highly significant effects of slaughter groups, treatment-group interaction, and amount of feed consumed. The regression of amount of feed eaten on liver cholesterol was negative, indicating that rabbits that ate more than the average amount of non-treated feed in addition to the treated feed had lower cholesterol levels. Since there was evidence that the treatments had different effects depending upon the slaughter group involved, no assessment of the differences among treatment means was made.

Heart cholesterol and predicted serum cholesterol were affected by treatment only. Statistical separation of the adjusted (least squares) treatment means for these 2 traits showed no evidence for a difference between the control and methionine groups nor between the cholesterol and cholesterol-plus-methionine groups. However, all other pairs of comparisons (cholesterol vs. no cholesterol) were highly significant.

Each aorta was examined both grossly and histologically for evidence of atherosclerosis. Several animals in both the cholesterol group and the cholesterol-plus-methionine groups showed histological evidence of atherosclerosis early in the experiment. Lesions were not grossly apparent, however, until the third slaughter period. All animals in both the cholesterol and cholesterol-plus-methionine groups developed grossly apparent atherosclerosis after the third week of supplementation. No apparent difference in the extent or degree of atherosclerosis could be observed between the rabbits given cholesterol or cholesterol-plus-methionine supplements. No instances of atherosclerosis were observed in the control or methionine group until the last slaughter period when one individual in each group was found to have developed the disease. This was due perhaps to a factor related to aging. The serum cholesterol level of neither animal was elevated yet atherosclerosis had developed.

The atheromatous plaques or lesions appeared first in the thoracic portion of the aorta, with the greatest concentration close to the thoracic arch. The lesions appeared as minute, yellow-white opaque flecks or nodules, raised slightly above the surrounding normal aorta. The confluence of a series of nodules resulted in the appearance of irregular streaks and patches, con-

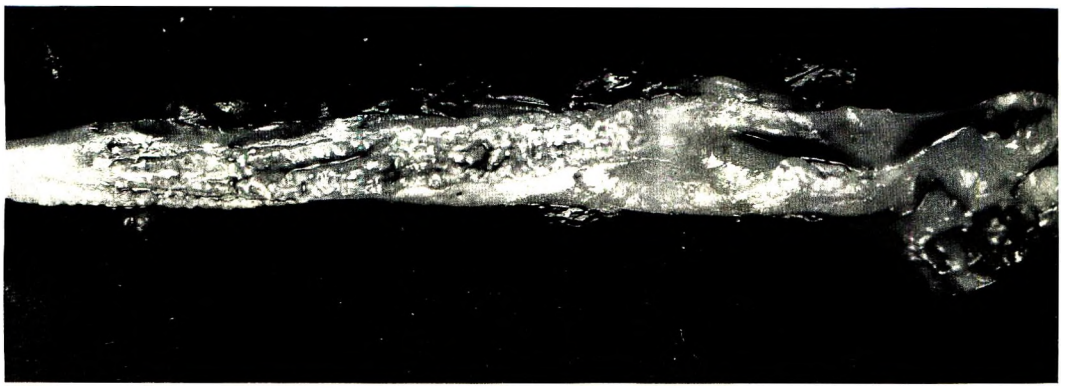

Fig. 1 Atheromatous plaques, thoracic aorta. Normal acrtic tissues at both extremities.

siderably more yellow than the smaller nodules (fig. 1).

The histological appearance of the lesions was that of a greatly vacuolized deposit, capping the aorta and protruding into the lumen. It consisted primarily of many lipid-filled foam cells and spindle-shaped cells scattered throughout a greatly thickened tunica intima. The upper third of the lesion, closer to the lumen, demonstrated a high degree of organization. The lower two-thirds, closer to the tunica media, showed evidence of extensive vacuolization and necrosis with an increase in intracellular ground substance. A loosening of fibers in the tunica media could be observed only in the areas beneath the plaque. The fibers were in the process of splitting and fraying and loss of cell outline was apparent. This appears to support the theory of Adams and Tugan (14) and Zugibe and Brown (15) that changes in the media may have an effect on the development of the atherosclerotic lesion. At the junction of the intima and media the foam cells were greatly enlarged and the deeply staining nuclei pushed to one side. Many small collagen and fibrin fibrils could be seen ramifying within the extensive amorphous intracellular ground substance. Located above this area and closer to the lumen were many spindle-shaped cells with deeply staining nuclei. The foam cells located in the outer portions of the plaque had not undergone the extensive vacuolization of those located more deeply in the lesion and retained a greater semblance of cell regularity.

Since no apparent difference in either the extent or degree of atherosclerosis could be observed, when comparing the cholesterol and the cholesterol- and methionine-supplemented rabbits, it is doubtful that a diet rich in methionine would be of value in relieving either hypercholesteremia or atherosclerosis unless the amount of dietary cholesterol is reduced.

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Effect of Manganese Deficiency on the Acid Mucopolysaccharides in Cartilage of Guinea Pigs [1,2]

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ABSTRACT Skeletal abnormalities observed in the manganese-deficient guinea pig at birth were studied from the standpoint of defects in biosynthesis of the organic matrix of cartilage during fetal development. When the adult female was provided with less than 3 ppm of Mn throughout gestation there was a significant reduction in the concentration of all acid mucopolysaccharides (AMPS) tested in rib and epiphyseal cartilage. The mixture of chondroitin sulfates which makes up the major constituent of the total AMPS present was significantly reduced, with chondroitin 4-sulfate and chondroitin 6-sulfate being affected about equally in epiphyseal cartilage. Chondroitin 6-sulfate was significantly reduced in rib cartilage. Hyaluronic acid and heparin were likewise significantly lowerd in the case of manganese-deficient animals at birth. Skeletal defects associated with manganese deficiency are believed to be brought about by defective metabolism of cartilage matrix constituents.

A manganese deficiency is readily produced in the newborn guinea pig if a purified diet providing less than 3 ppm of this trace element is fed to the adult female throughout the total gestation period (1). Skeletal defects observed in this species at birth are of several types and are often severe. There is a shortening of the long bones, enlargement and malformations of the joints, and deviations in the shape of the skull. A defective development of the rib cage is also observed with anterior-posterior flattening of the chest and misshapen or missing ribs.

Early work by Caskey et al. (2) using chicks, and by Shils and McCollum (3), Wachtel and associates (4) and Hurley et al. (5, 6) using rats, clearly establishes this mineral as essential for normal skeletal development. The general pattern of the deformities observed in the manganese-deficient guinea pig at birth suggested a defect in cartilagenous tissue and stimulated an interest in the relationship of manganese deficiency to disorders of the tissues of mesenchymal origin.

Of the three major components generally recognized in connective tissue, cells, extracellular fibers, and the extracellular amorphous ground substance, the state of the ground substance is speculated to have a profound influence on the life and function of tissues. The composition of the ground substance was therefore investigated, beginning with the acidic mucopolysaccharides (AMPS) present. The principal compounds in the AMPS group which have been identified in connective tissue are: hyaluronic acid; chondroitin 4-sulfate (chondroitin sulfate A); chondroitin 6-sulfate (chondroitin sulfate C); dermatan sulfate (chondroitin sulfate B); and heparin. The precursors of these AMPS compounds are mainly hexosamine and hexuronic acid which have been studied in manganese-deficient poultry. Leach et al. (7) observed that in the manganese-deficient chick there was a lowered radiosulfate uptake in cartilage and a reduced concentration of total hexosamines and hexuronic acid. The reduction in total hexosamine content occurred primarily in the galactosamine fraction. In 1964 Everson and associates [4] reported similar

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[2] Presented in part the 50th annual meeting of the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1966.
[3] This paper includes portions of a thesis submitted to the Graduate Division, University of California (Davis Campus) by Huan-Chang Tsai in partial fulfillment of the requirements for the Ph.D. degree.
[4] Everson, G. J., W. DeRafols and L. S. Hurley 1964 Manganese deficiency in guinea pigs related to ground substance defect and electrolyte balance. Federation Proc., 23: 448 (abstract).

changes in manganese-deficient guinea pigs. There was a significant reduction of hexuronic acid and hexosamine in epiphyseal cartilage, rib cartilage, brain and cornea in the deficient animals at birth. Both studies revealed that a low level of dietary manganese caused a reduction of precursors of AMPS in cartilage but did not reveal whether the metal has a specific effect or a more general effect on the metabolism of AMPS.

In the present study the distribution of AMPS in intact tissue (rib and epiphyseal cartilage) has been examined to determine the influence of manganese deficiency.

EXPERIMENTAL

Young guinea pigs weighing approximately 400 g were obtained from commercial sources and were fed a pelleted commercial diet supplemented with greens until the animals became acclimated.[5] A laboratory-prepared pelleted purified diet was mixed with the stock ration for a period of one week during which time the proportions of the 2 rations were adjusted so as to get the animals to accept the purified diet without a major loss in weight. Male animals were fed the stock diet at all times except for short periods when they were put into mating cages. The ingredients of the purified diet were as follows: casein,[6] 30; cornstarch, 20; glucose,[7] 10.6; sucrose, 10; roughage,[8] 5; agar,[9] 5; salts,[10] 6; cottonseed oil, 5; potassium acetate, 2.5; magnesium oxide, 0.5; inositol, 0.2; choline chloride, 0.2; and vitamins.[11, 12] A concentrate of vitamins A and D, α-tocopheryl acetate, and vitamin B_{12} were added to the oil of the diet to aid satisfactory mixing. The ingredients were combined and mixed with 400 ml of distilled water/kg of dry diet. As the preparation began to solidify it was pressed into a hopper of an electric meat grinder to produce pellets. The feed was spread thinly on a table to dry at room temperature, using ordinary electric fans to speed up drying. The dried ration was put into plastic bags and stored at − 4°. All animals received demineralized distilled water, and ascorbic acid was given orally 3 times weekly. In the present study the manganese-low ration contained less than 3 ppm Mn, and control animals received 125 ppm Mn as the sulfate, which was included in the salt mixture.

Newborn guinea pigs were killed within 24 hours after birth and were selected from 7 typical litters for both ration groups. Birth weights ranged from 70 to 120 g and the average weight of control animals was similar to that of the deficient young. Rib and epiphyseal cartilage were dissected from 10 animals in each ration group. The tissues were cleaned free of muscle and tendons and were weighed in tared glass vials and immediately placed in dry ice. Samples were stored at − 4° until chemical determinations were made.

The thawed cartilage samples were homogenized to a finely divided product using a high-speed homogenizer[13] and a Potter-Elvehjem glass homogenizer. The samples were defatted by chloroform-methanol extraction and dried in a vacuum oven at 35 to 40°. The total dry defatted cartilage for individual animals was dissolved in 0.5 N NaOH. Steps in the purification and preparation of the tissues for fractionation were carried out according to procedures described by Schiller et al. (8, 9) and Allalout (10). An aliquot of prepared cartilage equivalent to approximately 30 mg of dry defatted tissue was applied to DEAE-Sephadex A-25 columns. Fractionation of AMPS was carried out according to the procedure described by Schmidt (11). Reproducibility of removing samples from the columns was satisfactory (93%). Recovery of standard mucopolysaccharides[14] was 95%. Dische's carbazole method was used to determine the amount of uronic acid present (12).

[5] Wayne Guinea Pig Diet, Allied Mills, Inc., Fort Wayne, Indiana.

[6] Casein (Purified) Nutritional Biochemicals Corporation, Cleveland.

[7] Cerelose, Corn Products Company, Argo, Illinois.

[8] Solka Floc, a cellulose product of Brown Company, 733 Third Avenue, New York.

[9] Agar was found to vary widely in manganese content. Sources used were chosen for low manganese concentrations.

[10] Salts in grams: $CaCO_3$, 300; K_2HPO_4, 325; NaCl, 168; $FeSO_4 \cdot 7HOH$, 25; $MgSO_4 \cdot 7HOH$, 28; KI, 0.4; $ZnCO_3$, 0.25; and $CuSO_4 \cdot 5HOH$, 0.3. The salt mix used for the manganese-supplemented diet contained in addition 4.0 g of $MnSO_4$.

[11] Vitamins were added in amounts to provide for each kilogram of diet the following: (in milligrams) thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; nicotinic acid, 200; biotin, 1; folic acid, 10; 2-methyl naphthoquinone, 5; *p*-aminobenzoic acid, 100; α-tocopherol, 100; also vitamin B_{12}, 50 μg; vitamin A, 6,000 IU; and vitamin D, 600 IU.

[12] We are indebted to Hoffmann-LaRoche, Inc., Nutley, New Jersey, for generous supplies of ascorbic acid and to Merck and Company, Rahway, New Jersey, for pyridoxine and vitamin B_{12}.

[13] Virtis "45" homogenizer.

[14] Standards of acidic mucopolysaccharides were kindly made available by Dr. J. A. Cifonelli, LaRabida Institute, University of Chicago, Chicago.

Determinations of the isomers of chondroitin sulfate were carried out by procedures used by Mathews (13), Granath (14) and Schmidt (15). Aliquots were determined for acetylhexosamine using the method of Reissig et al. (16). Simultaneously an aliquot was analyzed for total uronic acid. The amount of uronic acid present in the chondroitin 6-sulfate was calculated directly from the amount of acetylhexosamine found. The amount of chondroitin 4-sulfate was derived from the difference between the total uronic acid and that due to the chondroitin 6-sulfate fraction.

RESULTS AND DISCUSSION

Skeletal changes observed in manganese-deficient guinea pigs at birth are illustrated in figure 1. Changes in the shape of the skull were usually observed. The development of the chest was especially sensitive to an inadequate supply of manganese with deformities of the ribs or missing ribs occurring and anterior-posterior flattening. Malformations and enlargement of the joints were observed.

The pattern of distribution of AMPS in rib and epiphyseal cartilage of 10 healthy control guinea pigs at birth is shown in table 1. Hyaluronic acid, chondroitin sulfates and heparin were present in both sources of cartilage with the concentration of each being higher for rib cartilage. A mixture of chondroitin sulfates made up the major AMPS constituent in the 2 types of cartilage, 6.37% (± 0.209) for rib calculated on the basis of fat-free tissue and 1.47% (± 0.126) for epiphyseal cartilage. Hyaluronic acid is found in next highest concentration with rib cartilage containing 0.48% (± 0.047) for the dry fat-free tissue and epiphyseal cartilage, 0.195% (± 0.109). A small amount of heparin was also present in both sources of cartilage. Dermatan sulfate was not found.

TABLE 1

Acid mucopolysaccharides of rib and epiphyseal cartilage

Animal no.	Rib cartilage			Epiphyseal cartilage		
	Hyaluronic acid	Chondroitin sulfates	Heparin	Hyaluronic acid	Chondroitin sulfates	Heparin
	% uronic acid in dry fat-free tissue			*% uronic acid in dry fat-free tissue*		
			Manganese-deficient			
1	0.21	4.82	0.14	0.13	0.92	0.09
2	0.31	4.20	0.19	0.12	0.57	0.11
3	0.41	5.60	0.39	0.16	0.92	0.04
4	0.23	3.70	0.40	0.15	0.80	0.09
5	0.37	5.80	0.16	0.17	1.28	0.12
6	0.34	5.60	0.18	0.21	1.17	0.11
7	0.30	3.60	0.36	0.14	1.20	0.07
8	0.36	5.40	0.26	0.19	1.96	0.05
9	0.58	4.86	0.10	0.09	1.08	0.03
10	0.38	2.60	0.18	0.10	0.42	0.03
Mean ± SE	0.35 ± 0.032	4.50 ± 0.463	0.24 ± 0.036	0.146 ± 0.024	1.03 ± 0.136	0.07 ± 0.11
			Control: plus manganese			
1	0.27	6.07	0.28	0.16	1.52	0.10
2	0.34	6.90	0.59	0.17	1.32	0.14
3	0.49	5.70	0.75	0.24	1.04	0.14
4	0.62	5.50	0.36	0.20	1.09	0.14
5	0.37	6.50	0.21	0.25	1.10	0.17
6	0.40	6.20	0.53	0.16	1.30	0.11
7	0.64	6.60	0.80	0.22	1.80	0.14
8	0.58	5.60	0.34	0.18	2.28	0.10
9	0.52	7.38	0.44	0.16	1.40	1.12
10	0.76	7.20	0.40	0.21	1.88	0.10
Mean ± SE	0.48 ± 0.047 $P < 0.05$	6.37 ± 0.209 $P < 0.01$	0.47 ± 0.062 $P < 0.02$	0.195 ± 0.109 $P < 0.1$	1.47 ± 0.126 $P < 0.05$	0.126 ± 0.008 $P < 0.02$

Fig. 1 Cleared, alizarin red S-stained skeletons of control (right) and manganese-deficient (left) guinea pigs at birth.

The distribution of AMPS in the cartilages of manganese-deficient animals at birth differed quantitatively but not qualitatively from that in control animals (table 1). The amount of chondroitin sulfates present in the manganese-deficient animals was approximately 70% of that present in the manganese-adequate groups. This change was noted in both rib and epiphyseal cartilage. These observations are in agreement with the work of Leach et al. (7) which showed that in manganese deficiency there is a reduction of galactosamine in the organic matrix of cartilage. The present data further show that there is a highly significant reduction of hyaluronic

TABLE 2

Isomeric chondroitin sulfates of rib and epiphyseal cartilage

Animal no.	Rib cartilage		Epiphyseal cartilage	
	Chondroitin sulfate C	Chondroitin sulfate A	Chondroitin sulfate C	Chondroitin sulfate A
	% uronic acid in dry fat-free tissue		*% uronic acid in dry fat-free tissue*	
	Manganese-deficient			
1	2.89	1.93	0.36	0.30
2	2.10	2.04	0.30	0.25
3	4.06	1.62	0.28	0.62
4	1.90	1.58	0.62	0.19
5	3.42	2.72	0.80	0.46
6	3.30	1.92	0.76	0.26
7	1.62	1.68	0.80	0.42
8	2.92	2.42	1.30	0.66
9	3.06	1.68	0.26	0.18
10	1.36	1.28	0.64	0.50
Mean ± SE	2.66 ± 0.277	1.89 ± 0.134	0.612 ± 0.103	0.38 ± 0.055
	Control: plus manganese			
1	4.02	2.00	1.28	0.30
2	4.90	2.02	0.96	0.24
3	2.00	3.72	0.46	0.70
4	2.48	2.80	0.60	0.88
5	3.42	2.68	0.64	0.48
6	4.16	1.96	0.78	0.56
7	3.48	3.40	1.18	0.66
8	4.42	1.18	1.28	1.00
9	6.06	1.34	1.06	0.30
10	5.92	1.28	1.40	0.48
Mean ± SE	4.09 ± 0.377	2.24 ± 0.265	0.924 ± 0.122	0.56 ± 0.079
	$P < 0.05$	ns	$P < 0.1$	$P < 0.1$

acid in rib cartilage in deficient guinea pigs ($P < 0.05$). A slightly lower value of hyaluronic acid was found for epiphyseal cartilage ($P < 0.1$). The reduction in heparin in both rib and epiphyseal cartilage is highly significant in the manganese-deficient group ($P < 0.02$).

The influence of manganese deficiency on the distribution of isomers of chondroitin sulfate was studied. It will be observed from table 2 that both chondroitin sulfate C and A were present in both sources of cartilage. Chondroitin sulfate C was the major isomer observed in most cases, being present in a ratio of 1.6:1 with chondroitin sulfate A for epiphyseal cartilage and 1.8:1 for rib cartilage. Within a given group of 10 animals considerable individual variation was noted, and for which there is no explanation. Manganese deficiency caused a lowering of the amount of chondroitin sulfates C and A in epiphyseal cartilage and the degree of change was the same for the 2 isomers. For rib cartilage chondroitin sulfate C was significantly reduced in the case of manganese-deficient animals, whereas a slight difference in chondroitin sulfate A due to low dietary manganese was not statistically significant.

The present observations indicate a possible role of manganese for the biosynthesis of all AMPS tested. Manganese apparently is not involved in the metabolism of one special mucopolysaccharide such as is believed to be true for vitamin A which affects only sulfated mucopolysaccharides (17, 18).[15] The data suggest that manganese is involved in a more general step in metabolism essential for all AMPS. The skeletal abnormalities observed in the manganese-deficient guinea pig at birth are believed to be related to flaws in the metabolism of cartilage matrix.

[15] Goldberg, A., and G. Wolf 1965 The effect of vitamin A deficiency of hyaluronic acid, heparin, and chondroitin sulfate. Federation Proc., 24: 355 (abstract).

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Anomalous Development of Otoliths Associated with Postural Defects in Manganese-deficient Guinea Pigs [1,2]

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ABSTRACT The high incidence of abnormal head movements observed in manganese-deficient guinea pigs at birth has led to a study of structural and histochemical differences in portions of the ear of deficient and control animals. Oil of wintergreen preparations of the ear have been made and have revealed the failure of otolith development in the utricular and saccular maculae in manganese-deficient young. Post-natal supplementation with manganese has not reversed the symptoms of vestibular disturbances and supplemented animals have always shown the existence of unilateral or bilateral defects of the otoliths. A smaller proportion of the deficient animals have in addition abnormal curvatures of the semicircular canals and misshapen ampullae. Macular preparations of the ears were stained with metachromatic techniques to estimate the concentration of acid mucopolysaccharides present. Positive tests for AMPS were observed only in normal otolithic. These studies are a continuation of an investigation of the importance of manganese in AMPS synthesis and suggest that the defects of motion observed in deficient guinea pigs are brought about by faulty otolith development influenced by limitations in AMPS synthesis in the presence of manganese deficiency during fetal development.

The majority of the young of female guinea pigs maintained throughout pregnancy with a diet deficient in manganese are ataxic at birth (1). The affected animals show a lack of ability to maintain their balance when standing. They hold their heads in such extreme retraction with their forelegs in such rigid extension that they often fall over backwards. Walking movements are accompanied by incoordination and lateral tilting of the head. Some degree of adaptation occurs as these animals age, and they are able to move about more normally. Head tilting and retraction are, however, characteristically present throughout life. Supplementation with manganese at any period, and for any length of time postnatally does not bring about a reversal of these symptoms. Observations of similar defects of equilibrium in the young of manganese-deficient female rats have been reported by Hurley and Everson (2). In collaboration with C. W. Asling (3), abnormalities of ossification of the otic capsule and deformities of the semicircular canals and ampullae have been described.

The present paper reports additional studies of the ear of the manganese-deficient guinea pig involving in particular those structures of the ear known to be related to postural equilibrium. Evaluations of behavioral abnormalities were correlated on an individual basis with the anatomical morphology of the semicircular canals, their ampullae, and the otoliths of the utricular and saccular maculae.

EXPERIMENTAL

MATERIAL AND PROCEDURES

The animals used in this investigation were produced by using the procedure described by Tsai and Everson (4). Twenty-five control animals, representing 13 litters were examined. Thirty guinea pigs from 14 litters of unsupplemented deficient animals, and 7 deficient young from 6 litters which received postnatal supplementation with manganese comprised the deficient groups. Postural defects were scored by observation of behavior of animals allowed to move freely on a flat table top. Table 1 illus-

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TABLE 1

Occurrence of postural defects and otolith abnormalities

Dietary regimen	Age group	No. of animals	No. of litters	Postural defects		Otolith morphology						Abnormal shape of ampullae and semicircular canals	
						Absent bilaterally		Absent unilaterally [1]		Abnormal bilaterally		Abnormal unilaterally [1]	
				No.	%	No.	%	No.	%	No.	%	No.	%
Control	newborn	17	6	0		0		0		0		0	
Control	2–8 months	8	7	0		0		0		0		0	
Manganese-deficient	newborn	21	8	12	57	4	19	8	38	2	10	—	1 5
Manganese-deficient	2–8 months	9	6	6	66	2	22	4	44	1	11	2	22 2 22
Manganese-deficient, supplemented	4–10 months	7	6	5	71	2	28	3	42	—	—	—	— 1 14

[1] Normal contralaterally.

trates the distribution of the animals in these experimental categories.

At the time the animals were killed the otic capsules of both ears were dissected away from the adjacent skull bones and cleaned free of muscle, skin, tendons and fascia. Using a dissecting microscope for magnification, the portion of the medial aspect of the temporal bone overlying the saccular and utricular portions of the membraneous labyrinth was removed. This allowed visualization of the crystalline otoliths of the sacculus and utriculus, and their presence or absence was observed and recorded for all ears at this time.

One ear from approximately 20% of the animals was tested in the fresh unfixed state for the response of the otolithic and macular structures to metachromatic stains. The techniques used were those of Bélanger and Hartnett (5) using toluidine blue, and Gurr's thionin technique (6). In either case, the staining solution was applied directly to these membranes in situ using a fine tipped glass pipette. The tissues immersed in the stains were carefully dissected free from their bony attachments and removed to watch glasses containing the recommended rinsing solutions to prevent the loss of metachromasia. The specimens were then transferred to either Abopon or dilute glycerine jelly for photography.

The methyl-salicylate clearing technique described by Mayer (7) was applied to both of the ears of approximately 60% of all the animals, and to one ear in each of the remaining 40%. The fixation in formalin recommended by Mayer was found to be unnecessary, and the ears were fixed and dehydrated in ethyl alcohol with subsequent clearing in wintergreen oil. In the cleared otic capsule the bony structures are rendered translucent, and it was therefore possible to visualize the curvatures, diameters, orientation and relationships of the semicircular canals and their ampullae. In these same preparations, the crystalline granules of the otolithic membranes appear as brilliantly refractile masses when viewed with the aid of an obliquely directed stream of light. Similar refractile materials are also present in the ligament holding the foot plate of the stapes in the foramen of the fenestra ovale; in the articular liga-

ments of the ossicles; and in a strip which transects the malleus. The presence, relative amount and location of the refractile material in these varied positions were assessed in the preparations cleared in methyl salicylate.

The remaining individual ears taken from about 20% of the animals were used for histological examination. In these ears the medial plate of the temporal bone was broken as previously described, and in addition the bulla tympani was excised and removed. The stapes was then lifted from the oval window, and the bony septum above the vestibule, together with the apical and basilar coils of the cochlea were dissected away. The ear was immersed in Bouin's fixative, and air bubbles which would impede penetration were flushed out of the bony structures with a stream of Bouin's from a glass syringe fitted with a thirty gauge needle. Some of these specimens were allowed to fix in Bouin's for about 10 days. This brought about a mild decalcification and enabled the tissue to be sectioned in paraffin at 15 μ. Hematoxylin and eosin stained sections were examined for general morphology. The remaining specimens were fixed for only 4 to 6 hours in Bouin's in situ. Then the crista ampullae and the saccular and utricular membranes were dissected from their bony attachments, and were dehydrated, cleared and embedded. Sections of these tissues were cut at 5 μ and stained with the metachromatic techniques referred to previously. Alternate sections were stained with hematoxylin and eosin.

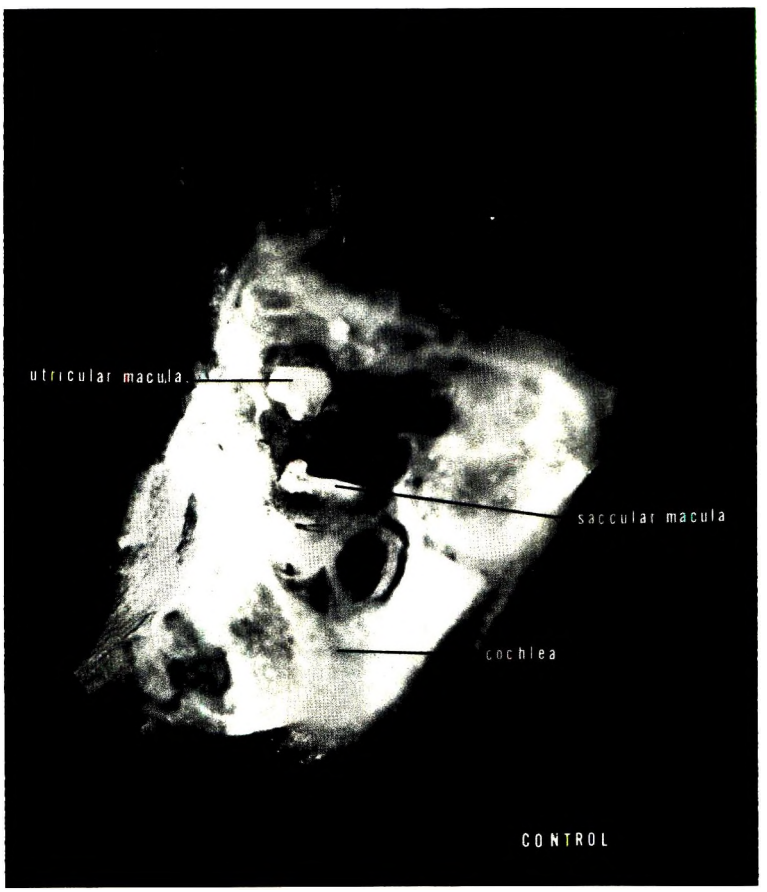

Fig. 1 Methyl salicylate preparation illustrating normal otolith structures of control animals.

RESULTS

A summary of the incidence of postural defects, types of otolith abnormalities, and of deformities of the semicircular canals and ampullae is presented in table 1. Postural defects were observed in none of the control animals. Of the unsupplemented deficient animals, 60% had observable postural abnormalities, whereas 71% of the deficient animals that had received postnatal supplementation with manganese showed head tilting or retraction.

In about 11% of the manganese-deficient guinea pigs examined, flattened or laterally distorted curvatures of the semicircular canals were observed. In each case the ampullae of these canals were also misshapen, appearing to flare more widely at the base and to be less symmetrically conical than normal. In all cases where such deformities were observed otolith abnormalities were also present.

Otoliths were scored in both the fresh and cleared whole preparations, and in the sectioned material as being normal, absent or abnormal. Figure 1 illustrates the normal otolithic structure present in the ears of all of the control animals. Comparable structures were present in all ears of deficient animals scored as normal. The "absent" category refers to ears in which no refractile material could be observed in the otolithic membrane of either the sacculus or the utriculus, in either the whole mount preparations or the sectioned tissue. Figure 2 represents the condition referred to as "abnormal." In these cases minimal amounts of calcium crystals could be observed to give a hazy appearance to the membranes in the cleared preparations.

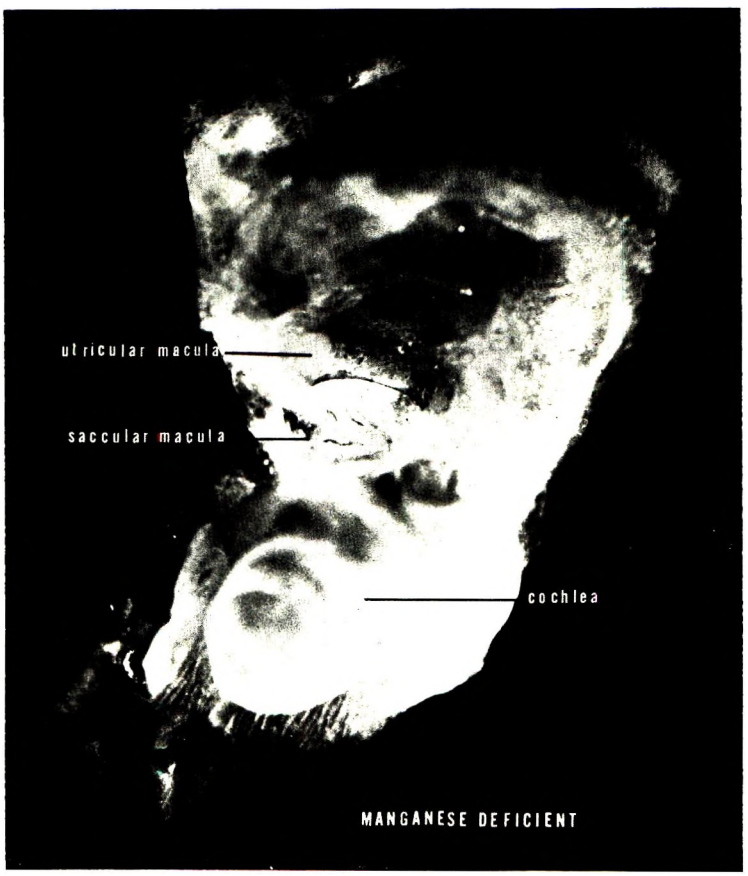

Fig. 2 Methyl salicylate preparation of the ear of a manganese-deficient guinea pig showing abnormal otoliths.

In sectioned material crystals could be identified, but were much reduced in number. In the defective guinea pig, both the saccular and utricular otoliths were involved, and to the same degree. The maculae of these structures were not observed to be abnormal in shape, size, or location in the animals having abnormal or missing otoliths.

The otoliths of the control animals were normal in all the animals examined. Of the newborn manganese-deficient group, 57% of the animals had either bilateral or unilateral absence of otoliths. This figure corresponds to the percentage of animals classified as being posturally defective. Two additional animals were found to have bilaterally "abnormal" otoliths. These had been classified as having normal posture. In the older manganese-deficient animals 66% had postural problems, and again 66% proved to have either unilateral or bilateral absence of the otoliths. Similarly, 3 animals that had been considered to have normal posture, were found to have "abnormal" otoliths. One animal showed this condition bilaterally, whereas the other two had normal otoliths in one ear and abnormal otoliths in the other. In such conditions it appears that the otoliths may be morphologically abnormal but functionally adequate, for postural control

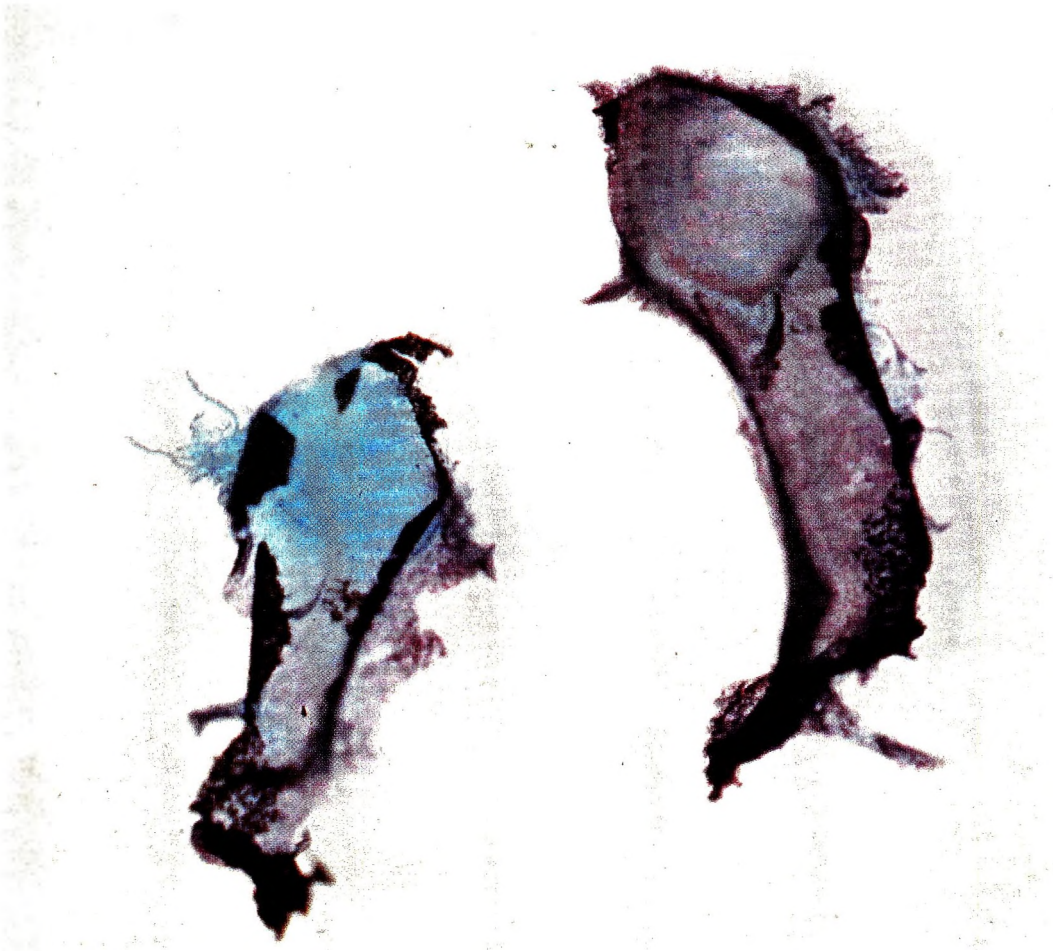

Fig. 3 Metachromatic reaction of otolithic membrane of control shown on right. Monochromasia of membrane of a deficient animal lacking otoliths on left.

under unstressed or familiar environmental conditions. The deficient animals that showed atypical head postures after receiving at least 3 months' supplementation with manganese all proved to have either unilateral or bilateral absence of otolithic crystals.

When the otolithic membranes and their maculae were stained as unfixed fresh preparations for the detection of mucopolysaccharides, a striking difference was revealed between the normal and defective groups. Figure 3 shows the metachromasia indicative of the presence of mucopolysaccharides in the membranes of the normal utricular otolith. The membrane preparation of a deficient animal in which the otolith was absent is shown to stain monochromatically. These observations were substantiated by the staining reactions of sectioned material. The absence of normal stainable acid mucopolysaccharides in the otolithic membrane of the manganese-deficient animals suggests an altered composition of the matrix.

Histologically the structure of the utricular and saccular maculae was found to be altered in the defective deficient animals. The normal and control maculae corresponded in all details to the illustrations and descriptions of Wersäll (8, 9). "The connective tissue plate clothed with the sensory epithelium, surmounted by a gelatinous membrane containing calcium crystals . . . and the hair-like processes of the stero and kinocilia which project from the sensory cells into the statoconium membrane" as described by Wersäll were readily identifiable in the control sections. In the sections of maculae from young deficient animals in which no otolithic crystals had been observed during dissection, the connective tissue plate appeared to be more compact, the neurepithelium appeared normal and the otolithic membrane could be identified lying on the tips of the hair cells. No crystalline granules were present in these membranes. In these preparations the connective tissue and the otolithic membrane responded monochromatically to the toluidine blue and thionin. In the oldest deficient animals examined histologically, additional changes appear to have occurred. The connective tissue plate was much reduced in thickness and very compact. The neurepithelium stained intensely and contained many cells with pyknotic nuclei. No hair cells were observed and no otolithic membrane could be identified. In these cases also, no trace of crystalline material was evident.

DISCUSSION

The equilibratory function of the utricular and saccular otoliths has long been recognized. The exact mechanism by which these structures operate has, however, been the subject of much debate. The techniques used to study the function of the otoliths have included abalation and destruction experiments, studies of the action potentials in the afferent nerves following stimulation of the receptor organs, and destruction of the efferent nerve supply to the maculae with subsequent recording of behavioral abnormalities. Such investigations have involved a wide range of species (fish, frogs, guinea pigs, cats and man) in which the structures present some variation in morphology. The resulting confusion in interpretation is easily understood.

The otolithic complex represents at least two morphological components; the otolithic membrane, the internal surface of which lies in contact with the hair cells of the neurepithelium, and the statoconia, or crystals of calcite, which coat the superior surface of the membrane. The defect in development which results from a prenatal deficiency of manganese in the guinea pig, has been shown in the present work to involve both of these components.

The prevailing view of the function of this complex is that it plays a role in the initiation of events within the hair-like processes of the neuroepithelial cells which ultimately results in the creation of a nerve impulse. This impulse is then transmitted to the receptors in the brain to indicate that a change in the position of the head has occurred.

Morphological studies of the ultra structure of the hair-like processes of the macular neurepithelium by Wersäll, Flock (10) and Smith (11) have shown that these consist of finely granulated protoplasm having a thin outer plasma membrane which is continuous with the plasma membrane of the cell. The free tips of

these sensory hairs lie within fine canals in the matrix of the otolithic membrane.

The composition of the matrix of the otolithic membrane has been studied in the pigeon ear by Dohlmann (12) and Ormerod (13), using ^{35}S uptake, electrophoretic and chromatographic techniques. They have demonstrated that sulpho-mucopolysaccharides are present in high concentrations in this structure. Analogous studies involving the histochemical identification of acid mucopolysaccharides in the axons of peripherial nerves have been reported by Abood and Abul-Haj (14). This group of investigators and others have postulated that mucopolysaccharides act as cationic accumulators. Dohlmann, in discussing the morphological and biochemical information relating to the macular otolithic membranes, states that "the hair cells lying in fine canals in the matrix of the otolithic membrane are soaked in a secretion containing two important constituent parts, the sulpho-muco-polysaccharides and potassium." He also suggests that "this material can and probably does act as a highly charged electrical field, surrounding an insulated entity containing electrically leading solutions." Any flowing movement induced in such a gel would, it is postulated, result in a movement of ions with resultant changes in electro-potential of the field.

The role of the crystalline material of the otolith has traditionally been conceived to be that of a weight on the tips of the hair processes. This theory implies that a change in the position of the head will cause the weighted hair to bend. Deformation or bending of the hair processes would then result in the stimulation of an electrical impulse. This theory has many opponents, and the lack of evidence of an anatomical connection between the crystalline particles and the hair tips makes the concept open to question. Flock (10), however, still writes of a sliding motion of the otolith during movement as being excitatory to the sensory hair cell. He offers no suggestion as to the mechanics of such excitation. The problem of statoconial function remains unsolved.

The present study, in which a prenatal dietary deficiency of manganese has resulted in otolithic abnormalities which can be equated with postural defects, has demonstrated that both components of the otolithic complex are irreversibly affected. Lyons has reported the relationship of postural defects to missing otoliths in mutant mice (15, 16). Erway and coworkers (17) have recently demonstrated that prenatal dietary manganese can effectively correct the expression of this genetic defect in the pallid mutant studied by Lyons.

The report by Tsai and Everson (4) that the mucopolysaccharide content of the epiphyseal cartilage is markedly reduced in the young of females fed a diet deficient in manganese throughout pregnancy indicates that the defect of metabolism of these substances is not confined to the membranous structures of the ear. That such defective metabolism could explain the malformations observed in the semicircular canals and their ampullae is highly possible, since these portions of the osseous labyrinth are first formed as cartilage, which later undergoes ossification. The same faulty metabolism may also be responsible for both the morphological and functional defects of the otoliths. The absence of a suitable matrix would thus prevent the deposition of crystalline calcite in which instance the otoliths could not be visualized in whole mount preparations or in sectioned material. The absence of adequate amounts of mucopolysaccharides in the matrix would also, according to the theories of Dohlmann (12), Ormerud (13), Abood and Abul-Haj (14), inhibit the initiation of transduction phenomena in the sensory hair cells.

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Steatorrhea in Rats with Intestinal Diverticula: Effects of changing dietary disaccharide and of coprophagy [1]

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ABSTRACT Thirty-two rats were studied for 20 weeks following surgery consisting of either the creation of a self-filling intestinal diverticulum, the creation of a self-emptying diverticulum, or a sham procedure. Rats with a self-filling diverticulum developed an impairment of fat absorption after 4 weeks following surgery when compared with rats in either of the other 2 groups. They could be studied with steatorrhea for at least a further 16-week period. Fat excretion did not differ significantly between the sham-operated rats and those with self-emptying diverticula throughout the study period. All animals were fed a diet in which a major source of carbohydrate was lactose. The substitution of sucrose for lactose, fed ad libitum did not alter fat excretion. Steatorrhea developed even when coprophagy was prevented by tail cups. Coprophagy did not alter the degree of impairment of fat absorption once steatorrhea was established.

The technique for the creation of diverticula in the intestine of the rat described by Cameron et al. (1) has been used extensively as a means of producing stasis within the intestinal lumen and intestinal malabsorption. The experiment to be described was undertaken to assess this preparation with respect to its suitability for long-term studies of steatorrhea. Also, the effects on fat excretion of a change in dietary disaccharide and of coprophagy and its prevention were measured.

MATERIALS AND METHODS

Thirty-two male rats of the Wistar strain (initial weight 150–200 g) were maintained in individual wire-bottom cages. Light and darkness were controlled automatically and the environmental temperature was maintained at 23 ± 2°.

Two semi-purified diets, differing only in carbohydrate content were fed ad libitum beginning 2 weeks prior to the onset of the experiment (table 1).

When the rats were first fed diet 1 (lactose), transitory diarrhea developed in many of them. This diarrhea never persisted for more than 2 weeks and therefore was not a problem during the experimental period.

Food intake was calculated by weighing the food offered to the rats each day and subtracting the weight of the food left plus that which was scattered.

The technique of Barnes (2) was used to prevent coprophagy and for the collection of feces throughout the experiment except for the period specified in the protocol. When tail cups were not in place, stools were collected from beneath the mesh cage bottom.

The collections of stools were made up to 250 ml in tap water and mixed thoroughly. Total fat content was assayed on a 10-ml aliquot by the method of VandeKamer et al. (3).

Hemoglobin was estimated on blood from the tail vein by the cyanmethemoglobin method (4).

The technique of Cameron et al. (1) was used to construct diverticula in the intestines of the rats. Under ether anesthesia, using aseptic techniques, single pouches of the self-filling or the self-emptying type were made, 7 to 8 cm in length, 10 to 15-cm distal to the pylorus. In another group, a sham procedure was carried out in which the peritoneum was opened and the intestine mobilized before reclosing.

Rats were fasted 12 hours pre-operatively and 72 hours post-operatively, after

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[1] Supported by a grant from the Medical Research Council of Canada.
[2] CIBA Medical Research Fellow 1965–1966.

TABLE 1
Composition of diets

	Diet 1	Diet 2
	g	*g*
Lactose	30	—
Sucrose	—	30
Dextrin	20	20
Casein	22	22
Corn oil	15	15
Salt mixture (12) [1]	4	4
Vitamin mixture [2]	4	4
Cellulose [3]	5	5

[1] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[2] The vitamin mixture contained: (in grams) vitamin A (200,000 units/g), 4.5; vitamin D (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine·HCl, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; (in milligrams) biotin, 2.0; folic acid, 9.0; vitamin B_{12}, 1.35; and dextrose to make 1 kg; obtained from General Biochemicals, Inc.
[3] Alphacel, General Biochemicals, Inc.

which feeding was re-started. The amount of diet offered was gradually increased over the next 3 days until the rats were given a full caloric intake. Water was given ad libitum throughout the study.

The operative mortality from the construction of a self-filling pouch was high. Seven of 16 rats died within a week of surgery. Of those with self-emptying pouches, only one of 12 died, whereas none of the sham-operated rats died. It was essential for survival that the blood supply to the bowel at the site of anastomosis be preserved. Operative mortality increased also, when the rats weighed less than 150 g.

Experimental plan

The 32 rats were studied in 3 groups (table 2) for 3 consecutive periods totaling 20 weeks, an initial 8 weeks to allow steatorrhea to develop following surgery, a 6-week period during which dietary carbohydrate was altered and a final 6 weeks for the assessment of coprophagy and its prevention, after which the rats were killed (table 2).

During the second period, the diets were changed for 2 weeks from the diet containing lactose (diet 1) to the one that contained sucrose (diet 2). Diet 1 was then resumed for the remainder of the experiment. The dietary change was staggered so that only half of the rats of each group were changed at one time. Thus animals within each group could act as controls for the other members of their own group.

Data were recorded as follows: 1) Bodyweight, tail-length and hemoglobin were measured weekly. Tail-length, measured to the closest 0.5 cm, was taken as an indication of skeletal growth. 2) Fecal fat excretion, expressed as a percentage of dietary intake was measured on 10 consecutive collections. Each covered a 2 week period. 3) Segments of intestine, approximately 2 cm in length were taken from the intestinal tract of each rat at the end of the experiment as follows: (a) jejunum, 2 cm proximal to pouch; (b) pouch orifice; (c) mid-pouch; (d) tip of pouch; (e) 2-cm distal to pouch; and (f) distal ileum. Sections were taken at comparable levels from the sham-operated group.

The segments were split longitudinally, spread on a card for correct orientation and to prevent curling. Tissue, with the card, was placed promptly in Bouin's solution for 4 hours after which the fixative was changed to 70% ethanol. Paraffin-embedded sections were stained with hematoxylin and eosin.

TABLE 2
Experimental plan

Period no.	Duration	Diet	Coprophagy
	weeks		
1	8	1, lactose	prevented
2	6	1, lactose (4 weeks) 2, sucrose (2 weeks) [1]	prevented
3	6	1, lactose	allowed (4 weeks) prevented (2 weeks)

[1] The trial of a sucrose diet occurred during the initial 2 weeks of period 2 in approximately half of the rats of each group, and during the second 2 weeks in the remainder. All rats were returned to the lactose diet for at least 2 weeks prior to period 3.

RESULTS

Survival. The rats that survived the first post-operative week also survived for the remainder of the experimental period. None of the rats died during the actual experimental period.

Weight gain. Mean body weights of each group continued to increase during the 20 week period as plotted in figure 1. Although the differences in weight gain between groups 1 and 3 after 20 weeks are significant ($P < 0.01$), the differences between group 2 and either group 1 or 3 are not significant after 20 weeks.

Tail length. Mean values for tail length, listed in table 3, showed a significant ($P < 0.01$) decrease in group 1 (self-filling pouches) compared with the other two.

Food intake. In each group, total intake increased significantly ($P < 0.001$) when rats were changed from the lactose feeding to the sucrose feeding and also when fecal collectors were removed. When the rats returned to the basal lactose diet or the tail cups were replaced, intake decreased to the previous level. Over the 20-week period of study there was a significant difference ($P < 0.001$) between the mean food intake of each of the 3 groups as shown in table 3.

Fat excretion. Mean fat excretion for each group of animals at fortnightly intervals is plotted in figure 2. The rats in group 1 with self-filling pouches developed significant steatorrhea. This excessive excretion of fat was highly significant when compared with that of rats with self-emptying pouches or that of sham-operated animals ($P < 0.001$) at 2-week intervals after the fourth week. No significant difference was noted between the 2 control groups, group 2 and group 3, during the same period.

Effect of changing dietary disaccharide. The mean values for each group, immedi-

TABLE 3
Tail growth and food intake

Group no.	Tail growth	Total food intake
	cm	*g/rat/week*
1	3.0 ± 1.5 [1]	87 ± 12
2	5.0 ± 1.0	107 ± 9
3	5.0 ± 1.0	124 ± 8

[1] Mean ± SD.

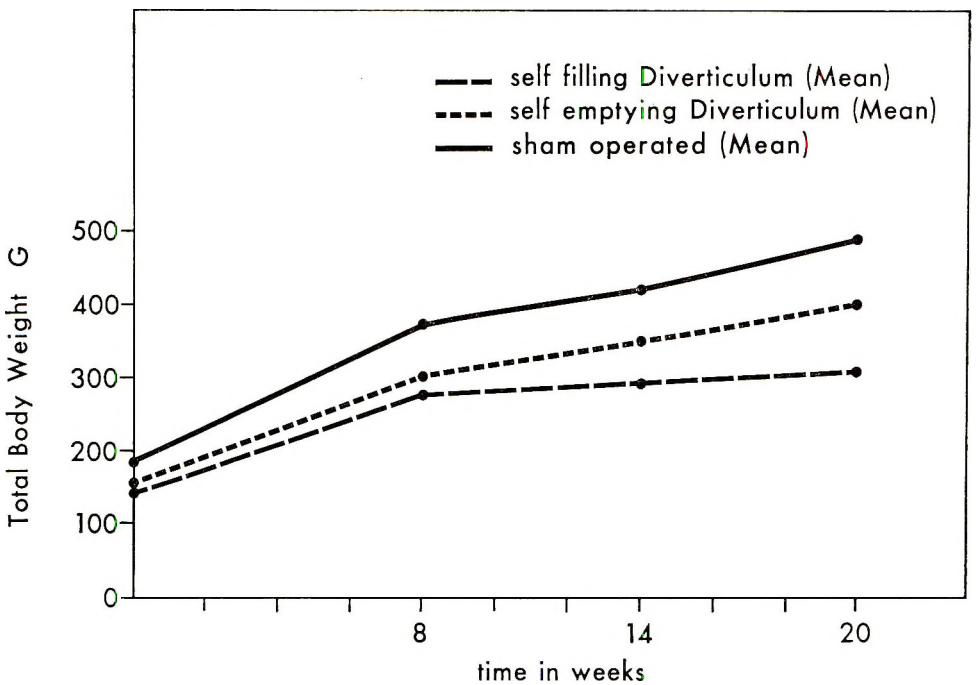

Fig. 1 Total body weight plotted as a mean for each group.

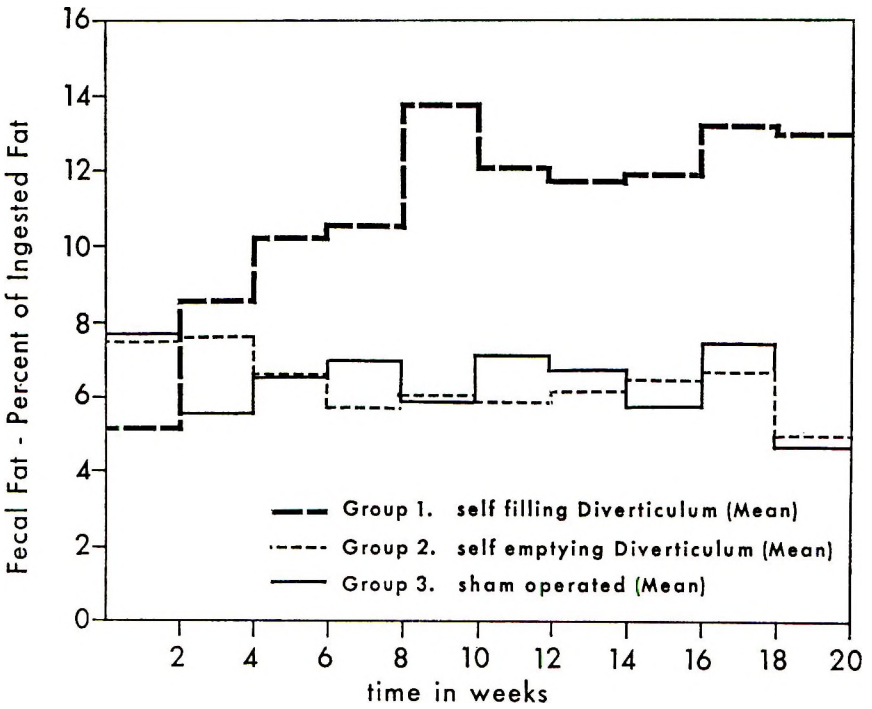

Fig. 2 Fecal fat excretion plotted as a mean for each group, consecutive 2-week total collections.

ately before, during and immediately after sucrose was substituted for lactose in the diet, are shown in table 4. No significant change occurred in any of the 3 groups.

Effect of coprophagy. The mean values for fat excretion, before, during and after the 4-week period when tail cups were removed, are presented in table 5. No significant changes were noted.

Hemoglobin. Mean hemoglobin levels for each group are plotted at intervals of 2 weeks in figure 3. Although the mean value for the sham-operated group increased over the 20 weeks, because of wide variation within each group, there was no significant difference between any of the groups at the end of the study ($P > 0.01$).

TABLE 4

Effect of changing dietary carbohydrate on fat excretion

Feeding	Fat excretion		
	Group 1	Group 2	Group 3
	% of ingested fat		
Diet 1, lactose	12.2±4.2 [1]	6.1±2.1	6.8±1.4
Diet 2, sucrose	12.8±4.5	5.7±1.7	6.1±1.4
Diet 1, lactose	12.2±5.6	5.9±1.8	6.6±2.8

[1] Mean ± SD.

Necropsy findings. Gross. Abnormalities were confined to the rats in group 1 with self-filling pouches. The pouches were enlarged to 2 or 3 times their original length and diameter and were filled with foul, turbid fluid. In five of the nine rats, altered blood was present in the pouches and in the small bowel distal to the pouch. No specific bleeding site was found. The remainder of the intestinal tract was normal. The pouches of rats in group 2, in contrast with those of group 1, were unchanged from their original size. The intestines of the sham-operated group were normal. The liver, spleen and pancreas were normal in all rats. The weights of the livers and spleens did not differ among the groups.

Microscopic. When sections of jejunal mucosa from rats with a self-filling pouch were compared with sections from rats of group 2 and sham-operated controls, abnormalities were confined to the pouches of group 1 (self-filling) in which the villi

TABLE 5

Effect of coprophagy on fat excretion

Consecutive 2-week periods	Coprophagy	Fat excretion		
		Group 1	Group 2	Group 3
		% of ingested fat		
Week 12–14	prevented	11.7 ± 5.6 [1]	6.2 ± 1.8	6.7 ± 2.6
Week 14–16	allowed	11.4 ± 4.2	6.5 ± 1.4	5.8 ± 1.6
Week 16–18	allowed	12.4 ± 5.2	6.7 ± 1.8	7.5 ± 1.9
Week 18–20	prevented	12.2 ± 4.5	4.9 ± 1.9	4.7 ± 2.1

[1] Mean ± SD.

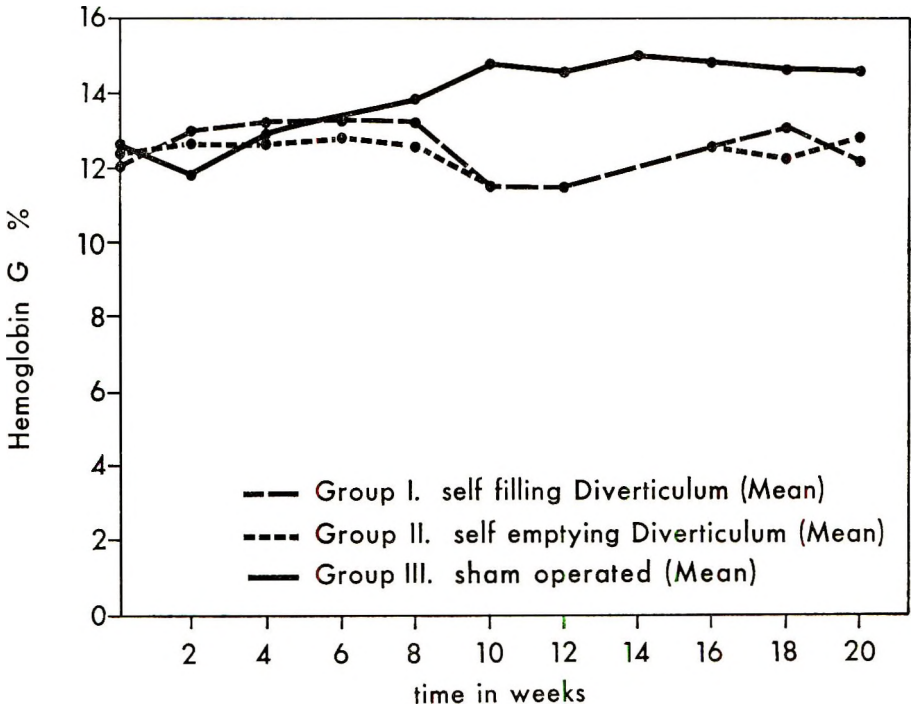

Fig. 3 Hemoglobin plotted as a mean for each group.

were regular but elongated. The mucosal structure of bowel both proximal and distal to the pouches was normal. The surface epithelium was regular and columnar at all levels and in all cases. The liver, spleen and pancreas were normal.

DISCUSSION

Under the conditions described, fecal fat excretion was significantly elevated in rats with self-filling diverticula of the proximal region of the intestine. This observation confirms the results of previous studies (5–7). Rats with self-emptying diverticula of the same initial dimensions, in which a comparable area of bowel was excluded from the continuity of the intestinal tract but without stasis, did not demonstrate altered fat absorption when compared with sham-operated ones. Exclusion of a segment of bowel from continuity of the intestinal tract, inherent in the technique, did not contribute to the altered fat absorption observed in the rats with self-filling diverticula. Furthermore, it appears unlikely that the procedure produced any significant trauma to the pancreas. The technique for the creation of self-emptying diverticula involved the same region of the bowel and that area was traumatized

to the same extent in both groups with impaired fat absorption. At autopsy, the pancreas was normal.

Death, ensuing upon the development of steatorrhea and presumably related to a profound anemia as described by Cameron et al. (1), and later by Toon and Wagenstein (8), did not occur in the present study. The rats did not develop anemia. Ellis et al. (9), using the same preparation, did not find severe anemia although their rats did not develop steatorrhea. Panish (6) described a survival of 3 to 12 weeks after surgery, inadequate for long-term study. Hoet and Eyssen (7) described some survivals of up to 15 months, although they studied their rats only for 5 weeks following surgery. The surgical technique used in the present experiment was identical to that used by previous workers. The particular strain of rats used in the present study conceivably might have influenced their survival.

There are 2 features in the methodology of the present study that do differ from previous reports, although the relationship to survival cannot be defined on the basis of the available data. The basal diet fed to the rats during the initial phase of the experiment contained lactose rather than sucrose, a disaccharide used more frequently in semipurified diets. The reasoning behind the use of this dietary sugar and the subsequent manipulation of disaccharide content of the diets in the present study warrants further explanation.

There is good evidence that the enteric microflora fulfill an important function in the pathogenesis of malabsorption associated with diverticula in the small intestine (10). The way in which these bacteria fulfill their role is much less clear. Although Donaldson (10) has shown that bacteria within intestinal diverticula may deconjugate bile salts, the significance of this mechanism has not been established.

The use of lactose as a dietary sugar and the assessment of a change to sucrose in the diet represents a preliminary attempt to study the fermentative activity of the microflora in relation to "blind-loop" steatorrhea. Lactose, in acute experiments, is absorbed less well than sucrose by the upper small intestine of the rat (11). In theory, fermentative activity in the diverticula should be increased with a lactose diet when compared with a sucrose diet. Proportionately more lactose than sucrose might reach the diverticulum to act as substrate for anaerobic glycolysis. In fact, changes in dietary sugar from lactose to sucrose and vice-versa failed to alter fat excretion significantly.

However, it cannot be concluded that the fermentative activity of the microflora does not influence fat excretion in the preparation studied. The rats fed ad libitum consistently ate more during the periods when they were fed the sucrose-containing diet than when given feedings containing lactose, potentially negating any effect of a qualitative change in dietary sugar. Studies are now in progress in an attempt to clarify this point. Also, the possibility exists that by basing fat excretion data on pooled collections of 2 weeks' duration a slight alteration in fat excretion was masked.

The second feature of the present study that differs from previous reports is that steatorrhea was produced in rats with intestinal diverticula while at the same time coprophagy was prevented by tail cups. Barnes has emphasized the significance of the role of coprophagy in the metabolism of the normal rat (2). The ingestion of fecal flora was not essential for the development of steatorrhea in the preparation studied. Steatorrhea developed less rapidly following surgery when compared with published series in which coprophagy was not prevented (6, 7). It was less severe than that reported by Panish (6) but the data are not strictly comparable. It is unlikely, however, that coprophagy prevention was a factor in permitting long-term survival. In the latter phase of the study, fecal cups were removed for 4 weeks, producing changes in neither general status, fat excretion, nor hemoglobin level.

The histological changes in the self-filling diverticula are in agreement with previous reports (5–7).

Considerable progress has been made in our understanding of certain mechanisms related to the pathogenesis of the intestinal malabsorption associated with intestinal diverticula. The relative significance of these mechanisms will not be elucidated until long-term studies can be undertaken

in the intact animal. The recognition of this experimental model of steatorrhea in the rat as suitable for long-term study during which the unknown influence of coprophagy can be eliminated, should be a useful adjunct to further studies in this field.

ACKNOWLDEGMENT

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Relationship between Serum Protein Level and Body Composition in the Chick [1,2]

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ABSTRACT Studies were conducted with 10-day-old chicks to determine the effect of varying the protein and energy intake on body composition, serum protein levels and the relationship between body composition and serum protein levels. Diets first-limiting in lysine as well as those calculated to be adequate in all the essential amino acids were used. Birds were pair-fed at each protein level to control the energy intake. Fat, moisture, and protein analyses were made on the carcasses of fasted whole birds at the end of a 15-day feeding period. Blood samples were drawn from the chicks not killed for the carcass composition analyses and analyzed for total proteins and albumins. When the dietary protein level was reduced without changing the energy level, both total serum protein and albumin levels were reduced. However, when the daily energy allowance was reduced without changing the protein intake, there was a rise in both total serum protein and albumin levels. There is a very high correlation between either total serum protein or albumin levels and body composition data. The data suggest that serum protein or albumin levels may serve as an index of body composition at a given age.

There has been considerable interest in the possible use of serum protein levels or the albumin-to-globulin ratio as a sensitive biochemical index for appraising protein nutritional status. Feeding low protein diets has been associated with decreased total serum protein and serum albumin levels in rats by Allison (1), in man by Scrimshaw and Behar (2), and in chicks by Leveille et al. (3) and Leveille and Sauberlich (4). Schendel et al. (5) used the albumin levels to predict the degree of protein depletion in kwashiorkor patients. According to Arroyave (6), plasma albumin values are usually lower in populations with a low socioeconomic standard of living and with a diet low in protein of good quality. However, Albanese (7) has cited cases where low protein intakes have not beeen associated with low plasma protein levels; in fact, above-normal levels have been observed in relatively undernourished individuals. Graham (8) found that inadequate caloric intakes of infants, although adversely affecting weight gain, favor the synthesis of serum albumin. Conversely, when the protein in the diet is apparently adequate, high caloric intakes which favor rapid gain and nitrogen retention can adversely affect the serum albumin.

Marked changes in body composition of chicks have been observed when the level of protein has been varied in diets with different energy levels (9–11). However, there is no specific information in the literature about the relationship between body composition and serum protein levels. In the present paper, evidence is presented which suggests that total serum protein or albumin levels may serve as an index of body composition at a given age when the protein of the diet is either adequate or deficient in a single amino acid.

MATERIALS AND METHODS

Triplicate groups of 8 Arbor Acre male chicks were used in all experiments. The chicks were reared in conventional chick battery brooder units with raised wire floors. Water was given ad libitum. A chick starter mash was fed from the time the chicks were one-day-old through the first 10 days. The 10-day-old chicks were

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[2] A portion of a dissertation presented by the senior author as partial fulfillment of requirements for the Ph.D. degree to the Graduate School of the University of Maryland.

starved overnight (approximately 16 hours), weighed and distributed according to weight so that each group would have the same range and mean weight.

A preliminary trial established that the diet used in experiment 1, shown in table 1, was deficient in lysine. Experiment 1 had a 4 × 4 factorial design with 4 levels of protein (25.2, 18.9, 12.6 and 6.3%) and 4 levels of energy (3704, 2981, 2262 and 1539 kcal ME/kg). The diets were formulated so that the percentage protein in the diet could be varied without changing the quality. The step-by-step reduction of 6.3% protein was achieved by removing 6.25% corn gluten, 5.00% soybean meal, 0.04% DL-methionine, 0.03% DL-tryptophan, and 0.06% glycine from the basal diet and substituting 10.80 and 0.62% glucose and sand, respectively, to keep the diets isocaloric. The energy levels were lowered by reducing the glucose and soybean oil content of the diet in increments of 13.33 and 3%, respectively. No inert filler was used and the amount of restricted diets to be fed was calculated. The chicks receiving the diets with 3704 kcal ME/kg were fed ad libitum and daily records were kept. The birds were pair-fed at each protein level to control the energy intake. The allowances for the energy-restricted groups were forecast on a daily basis. Any over- or underestimates were corrected at the next feeding.

TABLE 1

Composition of basal diets

	Experiment 1	Experiment 2
	%	%
Fish meal	—	2.00
Corn gluten (60% protein)	25.00	—
Soybean meal (50% protein)	20.00	48.00
Starch	—	30.00
Glucose [1]	40.00	—
Soybean oil	10.00	14.00
Limestone	0.80	0.90
Dicalcium phosphate	2.40	2.40
Trace minerals [2]	0.20	0.20
Salt	0.40	0.40
Potassium chloride	0.30	0.30
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.14	0.14
Choline chloride (25%)	0.75	0.75
Vitamin mix [3]	0.50	0.50
L-Arginine	0.15	—
DL-Methionine	0.15	0.30
DL-Tryptophan	0.10	—
Glycine	0.25	—
Ethoxyquin [4]	0.0125	0.0125

[1] Cerelose, Corn Products Company, New York.

[2] Delamix with 2% zinc, Limestone Products Corporation of America, Newton, New Jersey.

[3] Vitamin mix provided the following per kg of diet: (in IU) vitamin A, 6,600; vitamin D_3, 1,408; *d*-α-tocopheryl acetate, 22; (in milligrams) menadione, 4.4; riboflavin, 8.8; Ca pantothenate, 22; niacin, 44; folacin, 0.88; pyridoxine·HCl, 3.3; biotin, 0.22; ascorbic acid, 22; thiamine·HCl, 2.2, vitamin B_{12}, 0.022; procaine penicillin, 5.5; and coccidiostat (Amprol, Merck & Co., Rahway, N. J.), 500.

[4] Santoquin, Monsanto Company, St. Louis.

The design of experiment 2 was similar to that of experiment 1 except that the second diet shown in table 1 was used. It was calculated to be adequate in all the essential amino acids. In this study there were 3 levels of protein (25.6, 16.0 and 6.4%) and 4 levels of energy (3647, 2906, 2165 and 1424 kcal ME/kg). The protein level was reduced by substiuting glucose, 13.7%; cellulose,[3] 2.58%; and sand, 2.58% for soybean meal, 18.00%; fish meal, 0.75%; and DL-methionine, 0.11%. The step-by-step reduction of energy was achieved by removing 10% starch and 3% soybean oil from the basal diet.

After the chicks had been fed the experimental diets for 15 days, they were starved overnight (approximately 16 hours) and weighed. Half of the birds from each group were killed and frozen for carcass analyses. Blood samples were drawn by heart puncture from the remaining chicks. The blood was combined from the 4 birds in each group for total serum and albumin analyses, using AutoAnalyzer methods.[4] Blank corrections were run on all samples. Crystalline bovine albumin, fraction V,[5] was used as a standard.

The frozen carcasses were cut up into small pieces and ground together through a meat grinder. The ground carcasses of the fasted birds were analyzed for fat, moisture and nitrogen. Moistures were determined using the official AOAC method (12) for drying in vacuo at 95 to 100°. For fat analysis, the samples were first dried for 5 hours then extracted for 14 hours in a Goldfisch fat extraction apparatus with low boiling point petroleum ether. Nitrogen determinations were made by the Kjeldahl method.

[3] Solka Floc, Brown Company, Berlin, New Hampshire.

[4] Technicon Instruments Corporation, Chauncey, New York.

[5] Armour Pharmaceutical Company, Kankokee, Illinois.

TABLE 2

Effect of dietary protein (deficient in lysine) and energy on chick serum protein levels and body composition [1]

Protein	Energy	Wt gain	Total serum protein	Serum albumin	Albumin/ globulin ratio	Fat	Moisture	Protein	Nitrogen
%	*kcal ME/kg*	*g*	%	%		%	%	%	*% dry basis*
25.2	3704	287 ± 7.42 [2]	2.32 ± 0.09	1.20 ± 0.05	1.07	13.1 ± 0.59	66.3 ± 2.0	16.6 ± 0.47	7.90
25.2	2981	251 ± 4.55	2.46 ± 0.09	1.21 ± 0.02	0.97	9.4 ± 0.21	69.5 ± 0.61	18.2 ± 0.32	9.52
25.2	2262	202 ± 2.94	2.55 ± 0.09	1.31 ± 0.06	1.06	4.5 ± 0.03	72.1 ± 0.01	19.4 ± 0.03	11.17
25.2	1539	121 ± 5.92	2.64 ± 0.07	1.34 ± 0.06	1.03	1.8 ± 0.07	75.3 ± 0.02	19.3 ± 0.02	12.51
18.9	3704	174 ± 1.29	2.14 ± 0.09	1.04 ± 0.01	0.95	16.1 ± 0.88	63.9 ± 1.10	16.5 ± 0.24	7.31
18.9	2981	144 ± 1.41	2.38 ± 0.02	1.21 ± 0.04	1.03	9.9 ± 0.70	69.7 ± 0.64	17.3 ± 0.40	9.12
18.9	2262	122 ± 2.52	2.55 ± 0.17	1.25 ± 0.03	0.96	4.9 ± 0.19	72.8 ± 0.53	18.4 ± 0.22	10.81
18.9	1539	70 ± 2.45	2.69 ± 0.05	1.37 ± 0.06	1.04	1.9 ± 0.69	75.3 ± 0.02	19.5 ± 0.26	12.64
12.6	3704	101 ± 5.07	2.18 ± 0.11	1.01 ± 0.07	0.86	18.4 ± 0.56	61.7 ± 0.52	14.8 ± 0.33	6.21
12.6	2981	87 ± 1.29	2.23 ± 0.08	1.04 ± 0.08	0.87	13.8 ± 1.06	66.2 ± 1.21	16.3 ± 0.37	7.74
12.6	2262	71 ± 2.16	2.59 ± 0.10	1.24 ± 0.05	0.92	8.2 ± 0.72	71.1 ± 0.01	17.1 ± 0.22	9.43
12.6	1539	45 ± 2.24	2.69 ± 0.13	1.34 ± 0.08	0.99	1.8 ± 0.16	75.7 ± 1.10	18.0 ± 0.55	11.91
6.3	3704	44 ± 2.52	2.01 ± 0.05	0.77 ± 0.05	0.62	22.3 ± 1.30	59.4 ± 1.15	14.9 ± 0.08	5.88
6.3	2981	36 ± 2.83	2.13 ± 0.16	0.88 ± 0.04	0.60	17.3 ± 1.26	63.7 ± 1.57	14.6 ± 0.50	6.48
6.3	2262	22 ± 0.82	2.27 ± 0.17	0.99 ± 0.03	0.77	10.7 ± 0.70	68.7 ± 0.34	16.5 ± 0.29	8.44
6.3	1539	7 ± 0.82	2.29 ± 0.18	1.05 ± 0.11	0.85	2.8 ± 0.56	76.2 ± 1.73	16.7 ± 0.48	11.28

[1] Three groups of 8 male White Rock chicks used/treatment. One-half of the chicks were killed for carcass analysis.
[2] SE of mean.

RESULTS AND DISCUSSION

The results of experiment 1 are shown in table 2. Marked changes in body composition were observed. At each level of protein, the birds were pair-fed so that they all received the same amount of protein. Reducing the energy intake of the chicks at each protein level reduced the gain in weight. The chicks fed the lower protein diets consumed less feed. Feed consumption figures for the 25.2, 18.9, 12.6 and 6.3% protein diets were 33, 25, 20 and 17 g/day.

The fat content of the chicks on a wet basis was increased as the protein level was decreased in the isocaloric diets fed ad libitum ($P < 0.01$). There was also a corresponding decrease in moisture content and percentage nitrogen on a dry basis. As the energy intakes were decreased with chicks receiving an identical protein intake, carcass fat on a wet basis decreased. However, moisture and protein content on a wet basis increased. When energy intake was reduced at each level of protein intake, the nitrogen retention was markedly decreased.

Reducing the protein in the isocaloric diets fed ad libitum resulted in a decrease in body weight gain, total serum protein and albumin levels. However, when the daily energy allowance was reduced without changing the protein intake, there was a rise in both serum protein and albumin levels. These differences were found to be highly significant ($P < 0.01$).

Experiment 2 was undertaken to determine the effect of restricting energy with a diet calculated to be adequate in all the essential amino acids. The effect of the diets on serum proteins and body composition are summarized in table 3. Experiment 2 confirmed the observation made in experiment 1 that reducing the daily energy intake without changing the protein intake increased the serum protein and albumin levels. Marked differences were noted when the carcass analyses of the 2 studies were compared. In experiment 2, the birds tended to overconsume in relation to energy needs at the lower protein levels with a resultant increase in body fat. The average daily feed intake for the 25.6, 16.0 and 6.4% protein levels were 32.5, 38.6 and 28.4 g/chick/day. When 2 groups

TABLE 3

Effect of dietary protein (adequate lysine) and energy on chick serum protein levels and body composition [1]

Protein	Energy	Wt gain	Total serum protein	Serum albumin	Albumin/globulin ratio	Fat	Moisture	Protein	Nitrogen
%	*kcal ME/kg*	*g*	%	%		%	%	%	*% dry basis*
25.6	3647	344 ± 11.4 [2]	2.61 ± 0.13	1.23 ± 0.03	0.89	9.3 ± 0.51	69.4 ± 0.95	18.0 ± 0.14	9.39
25.6	2906	286 ± 1.5	2.64 ± 0.09	1.25 ± 0.03	0.90	5.8 ± 0.89	71.7 ± 0.82	18.8 ± 0.26	10.60
25.6	2165	205 ± 8.6	2.71 ± 0.06	1.28 ± 0.02	0.90	4.1 ± 0.46	73.0 ± 0.45	18.8 ± 0.20	11.13
25.6	1424	111 ± 7.0	2.94 ± 0.10	1.36 ± 0.03	0.92	1.4 ± 0.04	75.9 ± 0.12	18.9 ± 0.17	12.52
16.0	3647	335 ± 3.8	2.29 ± 0.06	1.06 ± 0.01	0.86	13.9 ± 1.20	65.3 ± 1.21	17.4 ± 0.15	8.00
16.0	2906	300 ± 2.0	2.39 ± 0.10	1.16 ± 0.04	0.94	11.2 ± 0.85	67.7 ± 0.69	17.6 ± 0.25	8.70
16.0	2165	241 ± 6.4	2.64 ± 0.08	1.28 ± 0.01	0.94	6.5 ± 1.38	71.6 ± 0.14	18.4 ± 0.06	10.34
16.0	1424	148 ± 2.4	2.84 ± 0.13	1.36 ± 0.06	0.92	2.1 ± 0.17	74.8 ± 0.40	18.9 ± 0.11	12.02
6.4	3647	124 ± 9.6	1.91 ± 0.03	0.70 ± 0.05	0.58	27.0 ± 0.68	56.0 ± 0.21	14.2 ± 0.44	5.16
6.4	2906	100 ± 3.0	2.06 ± 0.06	0.79 ± 0.05	0.62	20.1 ± 2.59	61.1 ± 2.10	15.3 ± 0.42	6.29
6.4	2165	80 ± 2.6	2.11 ± 0.03	0.86 ± 0.07	0.69	15.9 ± 1.59	64.2 ± 0.83	16.5 ± 0.32	7.37
6.4	1424	49 ± 2.6	2.60 ± 0.14	1.16 ± 0.03	0.81	5.3 ± 0.53	71.7 ± 0.68	18.2 ± 0.27	10.28

[1] Three groups of 8 male White Rock chicks were used/treatment. One-half of the chicks were killed for carcass analysis.
[2] SE of mean.

with similar fat percentages were compared, it was noted that the serum protein levels were also similar. On the basis of this observation, correlations between total serum protein, serum albumin or albumin-to-globulin ratio and percentage body fat, percentage body moisture, percentage body protein and percentage nitrogen (dry basis) were calculated in experiment 1. The correlations between total serum protein and body fat, body moisture, body protein and nitrogen (dry basis) were −0.89, 0.86, 0.89 and 0.90, respectively. The correlations between serum albumin and the same variables were −0.83, 0.79, 0.90 and 0.84. The correlations between the albumin-to-globulin ratio and the body composition data were not as high. Exceptionally high correlations were also obtained in experiment 2. The correlation between total serum protein and percentage body fat was −0.97. Similarly, the correlation between total serum protein and percentage nitrogen (dry basis) was 0.99. The correlations between serum albumin and the variables from the body composition data were also very high.

In view of the high relationship between total serum protein or serum albumin levels and the body composition data, it appears that the total serum protein levels may serve as an index of body composition at a given age rather than an index of dietary protein adequacy. This hypothesis will explain why the serum protein levels rise with caloric-deficient diets since under caloric restriction the percentage body fat decreases.

The data from experiments 1 and 2 were combined for further correlation analyses. The high correlations that were observed when each experiment was considered alone were also found with the combined data. Using the combined data, the correlations between total serum protein and percentage body fat, percentage body moisture, percentage body protein and percentage body nitrogen (dry basis) were −0.91, 0.89, 0.90 and 0.91. Similarly, the correlations between serum albumin levels and the same body composition data were −0.90, 0.88, 0.91 and 0.90.

The high correlations obtained using the combined data give further support to the hypothesis that total serum protein levels may constitute an index of body composition at a given age. In both experiments 1 and 2 the birds were the same age at the end of the studies.

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Zinc Interference with Copper Absorption in Rats

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ABSTRACT A study was made to determine the site or sites at which zinc interferes with the intestinal absorption of copper. ^{64}Cu was administered to all rats via ligated, in vivo, segments of duodenum, and zinc was administered either intraduodenally or intraperitoneally. High levels of zinc depressed the absorption of ^{64}Cu when both the zinc and the ^{64}Cu were placed directly into the isolated segment. If the ^{64}Cu was put into the intestinal segment and the zinc was given intraperitoneally, no depression in ^{64}Cu absorption resulted. However, the rats given intraperitoneal injections of zinc had tissue zinc concentrations that were comparable to those of rats given zinc intraduodenally. These results indicate that zinc does not depress copper absorption by first building up to critical levels in some non-intestinal tissue or tissues and, subsequently, interfering with copper absorption. Rather, the evidence indicates that the depression of copper absorption by high levels of zinc is mediated either in or on the intestine.

Copper metabolism can be influenced by a number of ions. In 1937, Sutton and Nelson (1) observed anemia, subnormal growth and reproductive failure in rats fed an excess of zinc. Since that time, the effects of zinc intoxication and the subsequent reversal of some of these effects by treatment with copper have been studied by numerous investigators (2–7). Despite the effort devoted to this problem, the mechanism by which zinc interferes with copper utilization remains obscure.

In a recent publication from this laboratory (8), we reported that the uptake of ^{64}Cu from an isolated, in vivo, intestinal segment was retarded by zinc. We concluded from this that zinc was interfering with the absorption process; however, the experiments reported in that publication yielded no information regarding the site at which zinc was exerting its influence. If the mechanism of zinc interference with copper absorption is to be determined, the location at which this interference takes place must be pinpointed. The experiments reported in the present paper were designed to answer this question.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used in these experiments. They were housed in stainless steel cages with raised wire floors and were fed a commercial laboratory ration [1] which contained 10 ppm of copper and 45 ppm of zinc. Rats weighing 200 to 350 g were used in a randomized block design and rats were allocated to blocks on the basis of body weight.

^{64}Cu was received as $Cu(NO_3)_2$ solution, and was diluted to a copper concentration of 2.5 μg/ml using either distilled water or a $Zn(NO_3)_2$ solution. Each rat received 0.4 ml of one of the resultant solutions which contained 1.0 μg of copper and either zero or 1.0 mg of zinc. The ^{64}Cu was put into an isolated, in vivo, duodenal segment approximately 7-cm long. The preparation of isolated, in vivo, segments has been described previously (8, 9). Zinc-treated rats received zinc either in the duodenal segment along with the ^{64}Cu or by intraperitoneal injection. When both ^{64}Cu and zinc were administered intraduodenally, they were given simultaneously. When ^{64}Cu was given intraduodenally and zinc was given intraperitoneally, the zinc was given either zero, 2 or 18 hours prior to administration of the ^{64}Cu.

Three hours after administration of the ^{64}Cu, rats were anesthetized with ether and decapitated. ^{64}Cu uptake by blood, heart, kidneys and liver was determined by counting each tissue. Disappearance of ^{64}Cu from the isolated segment was determined

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

by counting the excised segment and its contents. All counting was done in a Nuclear Chicago Model DS-202 well-type scintillation detector attached to a Nuclear Chicago Model 132 computer-analyzer. Subsequently, the tissues were digested and concentrations of copper and zinc were determined. These analyses were conducted using a Perkin Elmer Model 303 atomic absorption spectrophotometer equipped with the DCR-1 readout accessory.

Data were subjected to an analysis of variance and individual means were compared using a multiple range test (10). Statements of significance are based on odds of at least 19 to 1.

RESULTS

Data demonstrating the effects of zinc administration on the absorption of ^{64}Cu are presented in table 1. Either the percentage of the dose which was recovered in the four sample tissues or the percentage disappearance from the segment can be used as an index of ^{64}Cu absorption. By either criterion, the only zinc treatment that depressed ^{64}Cu absorption was the one designated ID-0, in which both zinc and ^{64}Cu were put directly into the isolated intestinal segment.

Zinc administration had some effects on the relative distribution of ^{64}Cu among the sampled tissues. Intraduodenal administration of zinc tended to decrease the relative proportion of ^{64}Cu in the liver and to increase the proportion in the kidneys. When zinc was administered intraperitoneally at the same time that ^{64}Cu was given intraduodenally, it increased the proportion of copper observed in the liver and decreased the proportion noted in the kidneys. This effect was not observed in our first experiments of this type (8); however, it has been a consistent feature of all of our more recent studies. The reason for this discrepancy is not known.

The results reported in table 2 demonstrate the effects of zinc administration on tissue zinc concentrations. Intraduodenal administration of zinc (ID-0) caused a significant increase in serum zinc levels. Rats given zinc intraperitoneally and concurrently with intraduodenal ^{64}Cu administration (IP-0), had serum zinc levels that were similar to those of rats given zinc intraduodenally. Zinc levels in the serum of rats given intraperitoneal doses of zinc either 2 hours (IP-2) or 18 hours (IP-18) prior to ^{64}Cu administration, did not differ significantly from the control levels.

Liver zinc levels were lowest in the control rats. Administration of this element by any of the methods used in these experi-

TABLE 1

Effect of method of zinc administration on absorption of ^{64}Cu

Method of zinc administration [1]	% of dose recovered in sampled tissues [2,3]	% disappearance of ^{64}Cu from segment
Control (no zinc)	22.6 [a 4]	50.6 [a]
ID-0	11.4 [b]	31.5 [b]
IP-0	20.7 [a]	45.9 [a]
IP-2	19.7 [a]	55.1 [a]
IP-18	22.7 [a]	53.0 [a]

[1] ID = intraduodenal; IP = intraperitoneal; zero, 2, and 18 indicate that zinc was administered to the rats simultaneously with ^{64}Cu, 2 hours prior to, or 18 hours prior to ^{64}Cu.
[2] Percentage of dose recovered in blood, heart, kidneys, and liver.
[3] Each value is the mean of 9 observations.
[4] Entries in any column that are not followed by the same letter are significantly different.

TABLE 2

Effect of method of zinc administration on tissue zinc concentrations [1]

Method of zinc administration [2]	Zinc concentrations				
	Serum	Erythrocytes	Heart	Kidneys	Liver
	μg/ml	*μg/ml*	*μg/g dry wt*	*μg/g dry wt*	*μg/g dry wt*
Control (no zinc)	4.7 [ab 3]	14.2 [a]	93 [a]	144 [a]	128 [a]
ID-0	9.6 [c]	14.6 [a]	91 [a]	123 [a]	146 [ab]
IP-0	8.4 [bc]	14.5 [a]	92 [a]	148 [a]	161 [b]
IP-2	5.2 [ab]	17.0 [a]	164 [a]	144 [a]	165 [b]
IP-18	3.4 [a]	14.1 [a]	106 [a]	149 [a]	195 [c]

[1] Each value in the table is the mean of 9 observations.
[2] ID = intraduodenal; IP = intraperitoneal; zero, 2, and 18 indicate that zinc was administered simultaneously with ^{64}Cu, 2 hours prior to, or 18 hours prior to ^{64}Cu.
[3] Values in any column that are not followed by the same letter are significantly different.

ments tended to raise the concentration of zinc in the liver. Zinc levels in the livers of the rats that received zinc intraduodenally did not differ significantly from those of the controls and were the lowest among the zinc-treated rats. All rats that received zinc by intraperitoneal administration had liver zinc concentrations that were significantly higher than those of controls. The zinc levels of the erythrocytes, heart and kidneys were not changed significantly by any of the treatments used in these studies.

The concentrations of stable copper in the sampled tissues also were determined. None of the treatments had any significant effect on copper levels of these tissues.

DISCUSSION

The primary objective of this study was to determine whether the zinc interference with copper absorption is mediated at the intestinal level or if it is a somewhat more indirect effect that is a consequence of high zinc levels in some non-intestinal tissue or tissues. Much of the discussion centers on blood serum and liver since both have long been recognized as key tissues in the metabolism of copper (11). However, the arguments that are developed could apply equally well to any tissue.

These studies confirmed our prior observation (8) that zinc impeded absorption of ^{64}Cu if both were placed in the intestinal segment. However, if ^{64}Cu was given intraduodenally and zinc was administered intraperitoneally, no depression in ^{64}Cu absorption occurred.

If zinc were exerting its depressant effect on copper absorption via the liver, the zinc might be expected to first build up to some critical level in this organ and then interfere with the copper absorption process. In these studies, the zinc content of the liver was higher in all of the intraperitoneally dosed rats (IP-0, IP-2, IP-18) than in the intraduodenally dosed rats (ID-0). Yet, intraperitoneal dosing with zinc had no effect on ^{64}Cu absorption, whereas absorption was significantly depressed by the intraduodenal zinc administration. This indicates that zinc did not depress copper uptake by interfering with copper metabolism in the liver.

Comparable results were obtained for blood serum. Serum zinc levels of rats that received both zinc and ^{64}Cu intraduodenally (ID-0) did not differ significantly from those of rats that received ^{64}Cu intraduodenally plus a concurrent, intraperitoneal dose of zinc (IP-0). Thus, it is possible to use reasoning similar to that applied to the liver data, that is, if serum were the tissue at which zinc exerts its effect, the zinc level of serum would build up to some crucial concentration at which zinc would interfere with copper absorption. That was not the case in these studies.

The erythrocytes, hearts and kidneys of the intraperitoneally dosed rats were as high in zinc or higher than in those tissues of the intraduodenally dosed rats. Thus, the arguments that were used for serum and liver also apply to these tissues. These results indicate that zinc is not depressing copper uptake by building up in some non-intestinal tissue and, subsequently, interfering with copper absorption. Rather, the results of these studies support the thesis that this impairment of copper absorption is mediated primarily via the direct effects of zinc either in or on the intestine.

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Detoxication of Dietary Tannic Acid by Chicks [1]

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ABSTRACT In an effort to learn more about the mechanism for detoxication of dietary tannins in aves, tannic (digallic) acid (TA) was fed to young chicks at 0.5 and 1.0% of their diet together with supplemental methionine (as the hydroxy analogue, Ca salt) (MHA), choline, betaine, arginine and ornithine individually and in various combinations. In the absence of these nutrients, TA depressed the growth rate of chicks and the depression increased in severity with the level added and with duration of the feeding period. The TA-induced growth depression was partially alleviated by supplementing the diet singly with MHA or choline and to a lesser extent with arginine. The combination of MHA, choline (or betaine) and arginine (or ornithine) further reduced the ill effects of TA fed at the 1% level and completely remitted the effects of TA fed at the 0.5% level. With suboptimal levels of methionine, and in the absence of dietary TA, increasing increments of arginine depressed growth linearly. This was corrected when sufficient MHA was added. When TA was fed with suboptimal levels of methionine, the supplemental arginine had no effect on growth rate; but when MHA was added to the TA diet, supplemental arginine improved the growth rate, resulting in a significant reduction in toxicity of TA.

Grain sorghums containing relatively high levels of tannin were shown to be toxic to young chicks as measured by a reduction in growth rate and slightly elevated liver lipids (1). Tannic acid, per se, fed at a level equivalent to that occurring in the high tannin grain sorghum (1% of the diet) resulted in growth retardation of similar magnitude. Increasing the dietary level of choline and methionine corrected the growth inhibition caused by the high-tannin grain sorghum, but only partially alleviated that which was caused by feeding tannic acid. Booth et al. (2) presented evidence that the major metabolite in the urine of rats and rabbits ingesting tannic acid and gallic acid was 4-0-methyl gallate and concluded that the source of methyl groups for the 0-methylation of gallic acid included principally choline and methionine.

The objective of this series of experiments was to investigate the mechanism (s) for detoxication of tannins in chicks.

EXPERIMENTAL PROCEDURE

Several trials were conducted in which tannic acid (TA) [3] was fed to chicks at levels of 0, 0.5, or 1.0% of their diet. Choline, betaine, MHA,[4] arginine and ornithine were added to the diets singly and in various combinations at each level of tannic acid. The basal diet containing no added choline, methionine or arginine is shown in table 1.

TABLE 1
Composition of basal diet

	%
Yellow corn, ground	55.0
Soybean meal, solvent-extracted, dehulled	36.5
Corn oil	5.0
Limestone	0.5
Defluorinated phosphate	2.5
Salt (NaCl)	0.3
Trace minerals [1]	0.2
Vitamin mix [2]	+
Zn (as $ZnSO_4 \cdot 7H_2O$)	(10 ppm)
Calculated analysis:	
Protein, %	23.09
Metabolizable energy, kcal/kg	3204
Choline, mg/kg	1286
Methionine, %	0.365

[1] Contained: (%) Mn, 6.0; Fe, 2.0; I, 0.12; Co, 0.02; Cu, 0.2; and Ca, 26.5.
[2] Vitamin mix (per kg diet): vitamin A, 6600 IU; vitamin D_3, 880 ICU; *dl*-α-tocopheryl acetate, 2.2 IU; and (in milligrams) menadione sodium bisulfite, 2.2; vitamin B_{12}, 0.013; riboflavin, 4.4; pantothenic acid, 8.8; and niacin, 44.

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[1] Journal Paper no. 504 of the College Experiment Station, University of Georgia, College of Agriculture Experiment Stations.
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[3] Tannic (digallic) Acid (practical), Eastman Organic Chemicals, Distillation Products Industries (Division of Eastman Kodak Company, Rochester 3, New York).
[4] Ca salt of α-hydroxy methylmercaptobutyric acid (Monsanto Company, St. Louis).

One-day-old White Plymouth Rock cockerel chicks housed in electrically heated battery brooders were used in all trials. Triplicate pens of 10 chicks each made up each treatment except in trial 2 where duplicate pens were used, and they were fed the experimental diets from the first day of age for various periods from 18 to 28 days. They were weighed initially and at the termination of each trial. Feed conversion was based on weight gain and feed consumed during the entire experimental period. In previous trials no specific pathology could be found attributable to tannic acid toxicity, and hence chicks that died were not autopsied, although mortality records were kept.

RESULTS AND DISCUSSIONS

In the first trial an attempt was made to differentiate the effects of choline and methionine on TA toxicity. The basal diet was supplemented with choline and MHA at the levels shown in table 2 within levels of zero, 0.5 and 1.0% TA.

Growth was depressed significantly at the 1.0% level of TA but only slightly at the 0.5% level. When 0.09% MHA was added, growth was improved uniformly at all levels of TA and at all levels of supplemental choline. Thus, there was no apparent reduction in toxicity of TA at that level of MHA. When the MHA was increased to 0.18%, there was no further increase in growth rate at the zero and 0.5% level of TA but at the 1.0 level of TA growth was improved significantly. The result was a reduction in TA toxicity with the increased level of MHA. Increasing levels of choline within each level of MHA had no effect on growth rate at any level of TA.

In the second trial (table 3) betaine, choline and MHA were fed to chicks at the zero, 0.1 and 0.2% levels in a factorial design with zero and 1.0% added TA. The column headed "Difference" represents the difference in body weights of the chicks fed TA and those fed the basal diet without TA, expressed as a percentage of the latter in each case. Two values were omitted from this column in table 3 where the growth rate with the basal diet was unaccountably high or low resulting in an apparent growth depression which was unrealistic. In the absence of TA each of the supplements fed singly at any level (except the 0.1% added choline) appeared to give the maximal growth response with no further response from higher levels or combinations. In the presence of TA, however, the growth response to choline (or betaine) *and* MHA appeared to be additive. The growth depression caused by TA was reduced slightly by MHA at the highest

TABLE 2

Effect of choline, MHA [1] and TA on growth of chicks (trial 1)

Supplements		25-day body wt [2]		
		Tannic acid		
MHA	Choline	None	0.5%	1.0%
%	%	g	g	g
—	—	490 defg	473 bcde	419 a
0.09	0.045	546 i	505 efgh	448 ab
0.09	0.09	528 hi	518 ghi	464 bcd
0.09	0.135	533 hi	503 efgh	456 bc
0.18	0.045	524 hi	522 ghi	483 cdef
0.18	0.09	548 i	500 efgh	483 cdef
0.18	0.135	529 hi	509 fgh	479 bcdef

[1] Methionine hydroxy analogue, Ca (Monsanto Company, St. Louis).
[2] Values followed by the same letters are not significantly different ($P < 0.05$).

TABLE 3

Effect of MHA,[1] choline, betaine and TA on growth of chicks (trial 2)

Supplements			4-wk body wt [2]		Difference
			Tannic acid		
MHA	Choline	Betaine	None	1.0%	
%	%	%	g	g	%
—	—	—	616 b	442 g	28
—	0.1	—	600 b	498 def	— [3]
—	0.2	—	675 a	501 def	27
—	—	0.1	661 a	495 def	25
—	—	0.2	638 ab	467 ab	27
0.1	—	—	664 a	483 ef	27
0.1	0.1	—	665 a	551 cd	17
0.1	0.2	—	671 a	548 cd	18
0.1	—	0.1	647 ab	522 def	19
0.1	—	0.2	697 a	515 def	— [3]
0.2	—	—	668 a	514 def	23
0.2	0.1	—	657 ab	552 cd	16
0.2	0.2	—	675 a	548 cd	19
0.2	—	0.1	664 a	538 de	19
0.2	—	0.2	680 a	532 de	22

[1] Methionine hydroxy analogue, Ca (Monsanto Company, St. Louis).
[2] Values followed by the same letters are not significantly different ($P < 0.05$).
[3] Differences were unrealistic in view of the unaccountably low or high values on the basal diet in these instances.

level without added choline or betaine; whereas choline or betaine reduced TA toxicity only in the presence of supplemental MHA.

Because of the recognized mechanism for detoxication of benzoic acid by ornithine in avian species, the effect of supplemental arginine (trials 3 and 4) and ornithine (trial 4) on TA toxicity were tested along with that of choline and MHA (table 4).

In trial 3, choline appeared to be more effective in reducing TA toxicity than was MHA; however, graded levels of these nutrients were not used and therefore it was not possible to measure their relative need in the basal diet as compared with the TA diet.

A slight reduction in body weight occurred when the basal diet was supplemented with ariginine alone, and this was corrected when MHA and choline were added. Fisher et al. (3) showed that synthesis of muscle creatine increases with increased levels of dietary arginine. This in turn may cause a higher methionine requirement, since it is also required in the biosynthesis of creatine. In the presence of dietary TA, growth was actually improved when arginine was added. The combination of MHA, choline and arginine improved the growth of chicks fed TA significantly more than any one of these alone, resulting in a reduction in TA toxicity in a manner suggesting an additive effect of these nutrients.

Similar results were obtained in trial 4 with arginine and with an equimolar level of ornithine. Neither resulted in any improvement of the basal diet supplemented with MHA and choline but both significantly improved the same diets containing TA. In fact, the effects of 0.5% TA were completely overcome by the addition of MHA, choline and either arginine or ornithine.

In view of the arginine-methionine relationship observed in trials 3 and 4, two additional trials were conducted in which the basal diet was supplemented with MHA, arginine and TA factorially as shown in table 5. Increments of arginine added to the basal diet alone progressively reduced the growth rate of chicks resulting in a growth depression of the same magnitude as that caused by tannic acid when either were fed at the 1.0% level. Supplemental MHA corrected the growth depression caused by feeding arginine, but it did not appear to reduce the depression resulting from TA ingestion in the absence of supplemental arginine. This would be expected since the response to MHA supplementation of the basal diet alone (in trial 6) was linear, indicating that the minimal requirement of the chicks for methionine was not exceeded. Until the methionine response curve (in the absence

TABLE 4

Effect of choline, MHA, arginine and ornithine on tannic acid (TA) toxicity

Trial no.	Supplements to basal diet				4-week body wt [1]			Difference between 0–1.0% TA
					Tannic acid			
	MHA [2]	Choline	Arginine	Ornithine	None	0.5%	1.0%	
	%	%	%	%	g	g	g	%
3	—	—	—	—	618 ^{de}	—	442 ^{a}	29
	—	0.2	—	—	646 ^{ef}	—	505 ^{bc}	22
	0.2	—	—	—	662 ^{f}	—	482 ^{ab}	27
	—	—	1.0	—	583 ^{d}	—	465 ^{ab}	20
	0.2	0.2	—	—	666 ^{f}	—	529 ^{c}	21
	0.2	0.2	1.0	—	667 ^{f}	—	577 ^{d}	14
4	—	—	—	—	577 ^{ef}	493 ^{bc}	411 ^{a}	29
	0.05	0.05	—	—	620 ^{gh}	547 ^{de}	464 ^{b}	25
	0.10	0.10	—	—	649 ^{hi}	590 ^{efg}	484 ^{bc}	25
	0.20	0.20	—	—	664 ^{hi}	602 ^{fg}	494 ^{bc}	26
	0.20	0.20	1.0	—	668 ^{i}	651 ^{hi}	515 ^{cd}	23
	0.20	0.20	—	0.75	661 ^{hi}	625 ^{hi}	514 ^{cd}	22

[1] Values followed by the same letters are not significantly different ($P < 0.05$).
[2] Methionine hydroxy analogue, Ca (Monsanto Company, St. Louis).

TABLE 5

Effect of MHA and arginine on tannic acid toxicity (body weight)

Trial no. (and duration)	Supplements to basal diet		Body wt [1]		
			Tannic acid		
	MHA	Arginine	None	1.0%	Difference
	%	%	g	g	%
5 (18 days)	—	—	305 fg	247 ab	19
	—	0.25	284 def	244 a	14
	—	0.50	272 bcd	243 a	11
	—	0.75	255 abc	243 a	5
	—	1.00	245 ab	237 a	3
	0.1	—	339 h	276 cde	19
	0.1	0.25	333 h	289 def	13
	0.1	0.50	322 gh	289 def	10
	0.1	0.75	303 efg	304 efg	—
	0.1	1.00	310 fgh	297 defg	4
6 (21 days)	—	—	409 gh	328 a	20
	—	0.25	422 hi	343 ab	20
	—	0.50	405 fgh	322 ab	18
	—	1.00	366 cd	331 a	10
	0.1	—	439 ij	354 bc	19
	0.1	0.25	446 jk	378 de	15
	0.1	0.50	443 ij	397 efg	10
	0.1	1.00	444 ij	384 def	14
	0.2	—	469 k	372 cd	21
	0.2	0.25	457 jk	381 de	17
	0.2	0.50	458 jk	408 gh	11
	0.2	1.00	454 jk	406 fgh	11

[1] Values followed by the same letters are not significantly different ($P < 0.05$).

of TA) reached a plateau, it would not be expected that the chicks receiving TA would exhibit a greater response to MHA than those not receiving TA.

In the presence of dietary TA, growth was not reduced by supplemental arginine. As in trials 3 and 4, MHA and arginine appeared to have an additive effect in reducing the TA-induced growth depression. Feed-to-gain ratios obtained in trials 5 and 6 are shown in table 6. In all cases, feed consumed per unit of gain was greater for the birds receiving TA and in some cases actually exceeded feed consumed by the controls on a "per bird" basis. Thus the decreased body weight could not be attributed to decreased feed consumption.

These results indicate that both methionine and arginine (or ornithine) are involved in the detoxication of tannic acid in the chick. The function of methionine is probably to provide labile CH_3 groups for the production of 4-0-methyl gallate as proposed by Booth et al. (2) in work with mammalia. Potter and Fuller [5] have identified this compound (by means of paper chromatography) in the urine of hens fed tannic acid and gallic acid. In some instances, choline or betaine appeared to spare methionine in this function. The role of arginine or ornithine in detoxifying tannic acid is unknown and may be either direct or indirect. Vohra et al. (4) observed a significant reduction in nitrogen retention in chicks fed tannic acid, and a slight reduction in protein digestibility was noted by Chang and Fuller (1) in chicks fed high tannin grain sorghums. The astringent nature of tannins in precipitating soluble collagen protein may account in part for this effect; however, it appears more likely that the explanation lies in the detoxication mechanism involving ornithine.

Vohra et al. (4) were unable to correct the tannic acid-induced growth depression

[5] Potter, D. K., and H. L. Fuller 1965 Studies on the detoxication of tannic acid by chicks. Poultry Sci., 44: 1407 (abstract).

TABLE 6

Effect of MHA and arginine on tannic acid toxicity (feed/gain)

Trial no. (and duration)	Supplements to basal diet		Feed/gain	
			Tannic acid	
	MHA	Arginine	None	1.0%
	%	%	g	g
5 (18 days)	—	—	1.48	1.71
	—	0.25	1.53	1.70
	—	0.50	1.54	1.72
	—	0.75	1.58	1.70
	—	1.00	1.61	1.74
	0.1	—	1.39	1.54
	0.1	0.25	1.42	1.52
	0.1	0.50	1.43	1.53
	0.1	0.75	1.43	1.53
	0.1	1.00	1.48	1.56
6 (21 days)	—	—	1.56	1.74
	—	0.25	1.50	1.76
	—	0.50	1.54	1.74
	—	1.00	1.60	1.72
	0.1	—	1.47	1.63
	0.1	0.25	1.45	1.61
	0.1	0.50	1.46	1.57
	0.1	1.00	1.47	1.52
	0.2	—	1.42	1.55
	0.2	0.25	1.43	1.56
	0.2	0.50	1.41	1.51
	0.2	1.00	1.43	1.51

in chicks by supplementation with methionine, choline, betaine or ornithine. Their basal diet was relatively high in methionine and choline, however, which also would account for the relatively slight growth depression resulting from dietary TA.

Experiments are in progress to identify the metabolic end-products in the urine of hens fed tannic acid derivatives.

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Vitamin B_{12} Deficiency in the Golden Hamster [1]

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ABSTRACT A mild vitamin B_{12} deficiency, producing metabolic changes, was developed in the golden hamster using a diet consisting mainly of cornstarch and soybean protein. The deficiency was manifested by urinary excretion of appreciable amounts of methylmalonic and formiminoglutamic acids but no increase in excretion of aminoimidazolecarboxamide. The deficiency had no effect on body weight or hematological processes, at least for the duration of this study. Glutathione (GSH) concentration in the serum and liver was increased significantly in the deficient animals over that of the controls, but there was no change in the content of liver coenzyme A. The manifestations of the deficiency disappeared when the deficient animals were fed vitamin B_{12}-supplemented diets. Thyroid hormone-active compounds (thyroid powder and iodized casein) and a thyroid hormone-depressing compound (propylthiouracil) were added to some of the diets to determine whether these compounds would shorten or lengthen, respectively, the duration of time before the deficiency developed and to learn whether they exacerbated or alleviated the effects of the deficiency. No effect of either type of compound was noted.

The relatively few studies which have been made of the nutritional requirements of the golden hamster (*Mesocricetus auratus auratus*) indicate that, in general, the requirements are similar to those of other common laboratory animals (1). However, 2 groups of workers reported that there was no growth depression in hamsters fed vitamin B_{12}-deficient diets, nor could they find other signs of a deficiency (2, 3).

Vitamin B_{12} deficiency is usually induced in most other species by the use of diets containing mixtures of vegetable proteins or under conditions of physiological stress (such as the addition of iodized casein or thyroid powder to the diet or by exposure to a cold environment). The deficiency can be detected in most other species by measuring changes in urinary excretion or tissue levels of certain metabolites. For instance, methylmalonic acid (MMA) is excreted in the urine in this deficiency by humans, rats, and sheep (4, 5); formiminoglutamic acid (FIGlu) by humans, rats, and chicks (6–8); aminoimidazolecarboxamide (AIC) by humans and rats (9, 10). A decrease in soluble sulfhydryl compounds, mainly glutathione, in the liver and blood has been noted in this deficiency in rats (11).

An inverse relationship has been reported between tissue levels of vitamin B_{12} and pantothenic acid (or coenzyme A) in several species (12). Using dietary means for developing vitamin B_{12} deficiency in the rat, Boxer et al. (13) observed that coenzyme A levels in the liver were increased over those of the controls. Cold exposure of rats resulted in a reduction of erythrocytes, hemoglobin, and liver glutathione and in an increase in liver coenzyme A (12, 14). The levels were restored to normal by vitamin B_{12} supplementation (15).

A study of pantothenic acid deficiency in the hamster showed that there was a difference in the metabolism of this vitamin between the rat and hamster, in that the deficiency in the hamster did not prevent the accumulation of total fat and cholesterol in the liver when cholesterol was added to the diet as it did in the rat (16). Because of this difference in the hamster in response to pantothenic acid deficiency and the inverse relationship between tissue levels of vitamin B_{12} and pantothenic acid and because a vitamin B_{12} deficiency has not been reported in the hamster, the hamster appeared to be a

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useful animal for further studies on vitamin B_{12}.

The hamster is a hibernating animal, and response to cold stress differs from that of the rat; the hypertrophy and histological hyperactivity noted in the thyroid of the cold-stressed rat is not observed in the hamster (17). For this reason, it was thought that there might be differences in response to thyroid hormone-regulating substances. Therefore, two thyroid hormore-active compounds (iodized casein and thyroid powder) and a thyroid hormone-depressing compound (propylthiouracil) were added to some of the diets.

METHODS

As vitamin B_{12} deficiency in other species is not always reflected in depressed growth rates, a method was sought to indicate that a deficiency had developed. Hamsters excrete viscous urine which partially solidifies on the screen floor of the cage and consequently, it was not possible under our conditions to collect the total 24-hour urine excretion. Only the fluid urine which was collected was assayed, and it was assumed to be representative of the 24-hour excretion. It was reasoned, however, that if MMA, FIGlu, and AIC were excreted only by the deficient animals, a semiquantitative estimation of the excretion could be made. This assumption proved correct in a pilot study on a few animals, conducted prior to the study reported here. In the pilot study, appreciable amounts of MMA and FIGlu, but not AIC, were excreted by the deficient animals. The appearance of both MMA and FIGlu, not just one or the other, was used as indication that a deficiency had developed and the animals could be killed. MMA was noted in the urine at the end of 4 weeks and FIGlu at the end of 12 weeks of the experimental diets. Thirteen weeks were then established as the duration of the study.

Hamsters of both sexes, aged 3 to 4 weeks, were obtained from a commercial supplier.[2] For a period of 3 days they were fed a mixture of equal parts of ground laboratory ration[3] and cornstarch, as the pilot study had shown that the hamster adjusted better with a gradual change to a purified diet. At the end of 3 days, approximately 5 males and 5 females were assigned at random to each of 8 experimental diets. The animals were caged individually in standard-size metal cages with screen floors (0.6-cm mesh) and had access to food and water at all times.

The basal diet had the following composition: (in per cent) soybean protein, 25;[4] cellulose, 4;[5] cornstarch, 41.32;[6] powdered sucrose, 14; cottonseed oil, 10;[7] mineral mixture, 3.5;[8] choline bitartrate, 0.18; water-soluble vitamin mixture, 1;[9] fat-soluble vitamin mixture, 1;[10] thyroid powder (when added), 1;[11] iodized casein (when added), 1;[12] propylthiouracil (when added), 0.04.[13] Supplements were added as indicated in table 1.

Two consecutive 24-hour collections of the fluid urine were made from each animal, starting with the third week of the experimental diet. The first was assayed for FIGlu by the method of Tabor and Wyngarden (18) and AIC by the method described by McGeer et al. (19), with the modification that the urine was passed through an anion exchange column before color development.[14] The second urine sample was assayed for MMA according to the ether extract and chromatographic method described by Barness et al. (20), with the difference that the chromatogram was sprayed with bromphenol blue instead of bromcresol green. The ether extract was concentrated, and the spot in the chro-

[2] Simonson Animal Supply, Gilroy, California.
[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[4] Soya Assay Protein, General Biochemicals, Inc., Chagrin Falls, Ohio.
[5] Solka Flok. McKesson and Robbins, Inc., Chemical Division, Oakland, California.
[6] Colonial Food Supply of California, Oakland, California.
[7] Durkee Winterized Cottonseed Oil, Famous Foods, Berkeley, California.
[8] Mix UCB-1Rb, Williams, M. A., and G. M. Briggs (unpublished), contained in g salt (reagent grade)/21,000 g total mix: $CaCO_3$, 4350; $CaHPO_4$, 6780; Na_2HPO_4, 3906; KCl, 4380; $MgSO_4$, 1380; $MnSO_4 \cdot H_2O$, 92.4; $CuSO_4$, 7.8; ferric citrate (16.7% Fe), 90.6; $ZnCO_3$, 12.6; and KIO_3, 0.6.
[9] Contained/kg diet: (in milligrams) thiamine·HCl, 6.0; riboflavin, 6.0; pyridoxine·HCl, 6.0; folic acid, 2.0; Ca pantothenate, 10.0; niacinamide, 10.0; biotin, 0.2; menadione, 5.0; vitamin B_{12} (when added), 10.0 of 0.1% trituration; and granulated sucrose to make 10 g.
[10] Contained/kg diet: (in milligrams) vitamin A acetate distillate (500,000 IU/g), 20.00; vitamin D (irradiated ergosterol, 400,000 IU/g), 1.25; *dl*-α-tocopherol, 2.50; and cottonseed oil to make 10 g.
[11] Mann Research Laboratories, Inc., New York.
[12] General Biochemicals, Inc.
[13] Sigma Chemical Company, St. Louis.
[14] Modified in the laboratory of E. L. R. Stokstad, University of California, Berkeley.

TABLE 1

Body and liver weights of hamsters

Supplement to basal diet	Sex	No. rats	Body weights		Liver weights	
			Weaning	Final		
			g	*g*	*g*	*% body wt*
			Without vitamin B_{12}			
None	M	5	39 ± 4 [1]	99 ± 14	5.3 ± 1.1	4.4 ± 0.6
	F	5	34 ± 6	109 ± 17	5.0 ± 0.8	4.6 ± 0.2
1% thyroid powder	M	5	38 ± 6	116 ± 11	7.4 ± 1.4	6.4 ± 0.8
	F	5	37 ± 3	103 ± 10	6.0 ± 0.7	5.9 ± 0.9
1% iodized casein	M	7	37 ± 6	121 ± 17	8.0 ± 1.2	6.7 ± 1.0
	F	4	43 ± 4	105 ± 7	7.4 ± 0.5	7.3 ± 0.8
0.04% propylthiouracil	M	5	41 ± 2	116 ± 11	4.8 ± 1.2	4.1 ± 0.9
	F	5	39 ± 7	109 ± 4	4.4 ± 0.7	4.0 ± 0.6
			With 10 μg vitamin B_{12}/kg diet			
None	M	5	41 ± 6	109 ± 18	5.0 ± 1.2	4.6 ± 0.6
	F	5	38 ± 6	125 ± 14	5.7 ± 0.6	4.6 ± 1.0
1% thyroid powder	M	5	39 ± 6	123 ± 18	7.6 ± 1.7	6.1 ± 0.6
	F	3	44 ± 4	110 ± 5	6.6 ± 1.7	6.0 ± 1.8
1% iodized casein	M	5	41 ± 7	111 ± 18	6.8 ± 1.3	6.1 ± 0.5
	F	5	36 ± 9	96 ± 9	5.5 ± 0.5	5.8 ± 0.9
0.04% propylthiouracil	M	6	40 ± 6	106 ± 24	4.5 ± 1.2	4.1 ± 0.4
	F	5	41 ± 4	112 ± 13	4.8 ± 0.1	4.3 ± 0.5

[1] ± SD.

matogram represented the percentage of MMA in the 24-hour collection.

At the end of 13 weeks, 2 animals from each of the vitamin B_{12}-deficient groups were transferred to the corresponding vitamin B_{12}-supplemented diets. At the time of autopsy, all animals were anesthetized with sodium amytal, and blood was taken by heart puncture with a heparinized syringe. The liver was weighed (after the gallbladder had been removed). One portion of the liver was assayed for units of coenzyme A by the method of acetylation of sulfanilamide (21). Serum and another portion were assayed separately for reduced glutathione according to the method of Beutler et al. (22). Hemoglobin was determined by the cyanmethemoglobin method. Smears of blood and bone marrow from the entire shaft of the femur were stained with modified Wright's stain for the study of morphological changes. Hematocrit and red blood cell counts were made.

The results of the individual groups of animals were compared for statistically significant differences using Student's *t* test.

RESULTS

Growth and general appearance. There were no statistically significant differences in the weaning or final body weights among the groups of animals ($P > 0.05$) (table 1). Adult female hamsters are generally larger than the males, but this difference in body weight was not noted consistently in this study. Vitamin B_{12} deficiency did not affect the outward appearance of the animals, and any gross differences noted were in the animals fed the thyroid hormone - regulating compounds. Those animals given thyroid powder or iodized casein had rough-looking fur, were leaner in appearance, and at autopsy showed little body fat and large mottled livers, whereas those fed propylthiouracil had smooth-appearing fur, were plump in appearance, and had larger amounts of body fat and smaller livers than those fed the basal diets, whether or not vitamin B_{12} was present.

Urinary excretion of MMA, FIGlu, and AIC. The smallest quantity of MMA detectable was 22 μg, and all the animals fed the vitamin B_{12}-deficient diets excreted at least this quantity in 24 hours after receiv-

ing the diet for 4 weeks and every week thereafter. No MMA was detectable in the urine of any of the animals fed the vitamin B_{12}-supplemented diets.

FIGlu also was noted in the urine of only those animals that had been fed the vitamin B_{12}-deficient diets for 12 weeks. The value of urinary FIGlu for the thirteenth week of the study for the vitamin B_{12}-deficient animals ranged from 1.7 to 2.3 μmoles/24 hours.

No increase in the excretion of AIC by any animals was noted during the 13 weeks of the study. The amount excreted was 2 to 7 μg/24 hours for animals fed diets with or without vitamin B_{12}.

For those few animals that had been transferred from the vitamin B_{12}-deficient to the vitamin B_{12}-supplemented diets after 13 weeks, urinary excretion of MMA ceased after 3 weeks and FIGlu after 5 weeks of receiving the supplemented diets.

Blood parameters. Data on red blood cell counts, hematocrit, and hemoglobin are presented in table 2. There were no statistically significant differences between males and females in any groups; between parallel groups, for example, basal diet with and without vitamin B_{12}; or among groups of deficient or supplemented animals. Any variation in differential white cell counts or bone marrow smears were within the normal range.

The values for the blood parameters of the deficient animals transferred to the supplemented diets were within the range of the other animals.

Serum and liver glutathione. Table 3 presents the data on serum and liver reduced glutathione. Any differences noted in tissue levels were between the groups of animals with and without vitamin B_{12}. The deficient animals had somewhat higher serum and liver levels than the vitamin B_{12}-supplemented animals. Comparison of parallel groups shows that the differences in serum levels were significant at the 1% level for both sexes of all groups. The differences in liver content were not so consistent, being significant at the 1% level except between the males of animals fed the basal diets and between the males fed the diets supplemented with thyroid powder. No consistent differences due to sex within any groups were found.

The deficient animals that were transferred to the vitamin B_{12}-supplemented diets at 13 weeks showed a significant re-

TABLE 2

Red blood cell counts, hematocrit and hemoglobin

Supplement to basal diet	Sex	No. rats	Red blood cells	Hematocrit	Hemoglobin
			$\times 10^6$		*g/100 ml*
		Without vitamin B_{12}			
None	M	5	5.9 ± 0.6 [1]	45 ± 3	12.8 ± 0.4
	F	5	6.4 ± 0.9	45 ± 3	13.5 ± 1.8
1% thyroid powder	M	5	6.2 ± 0.5	46 ± 1	12.6 ± 0.5
	F	5	6.7 ± 0.3	46 ± 4	13.0 ± 1.4
1% iodized casein	M	7	6.6 ± 0.8	44 ± 4	12.5 ± 1.0
	F	3	7.2 ± — [2]	41 ± 1	11.8 ± 1.0
0.04% propylthiouracil	M	5	6.4 ± 1.0	46 ± 3	12.8 ± 1.4
	F	5	6.1 ± 0.5	45 ± 4	13.2 ± 0.9
		With 10 μg vitamin B_{12}/kg diet			
None	M	5	5.8 ± 0.6	48 ± 1	13.7 ± 1.2
	F	5	5.5 ± 0.4	48 ± – [3]	14.0 ± 0.9
1% thyroid powder	M	5	6.5 ± 0.5	48 ± 1	14.1 ± 0.5
	F	3	7.2 ± — [1]	46 ± 2	12.9 ± 0.9
1% iodized casein	M	5	6.8 ± 0.9	48 ± 1	13.6 ± 0.4
	F	5	6.6 ± 0.3	46 ± 2	14.3 ± 1.6
0.04% propylthiouracil	M	6	5.9 ± 0.5	49 ± 6	14.4 ± 1.0
	F	5	6.7 ± 0.5	47 ± 2	13.9 ± 0.6

[1] ± SD.
[2] SD not calculated because only 2 samples.
[3] SD less than 1.

TABLE 3

Serum and liver glutathione (GSH)

Supplement to basal diet	Sex	No. rats	Blood	Liver
			mg/100 ml	*mg/g*
	Without vitamin B_{12}			
None	M	5	31.9 ± 1.8 [1]	1.2 ± 0.4
	F	5	31.5 ± 2.4	1.4 ± 0.3
1% thyroid powder	M	5	33.2 ± 1.9	1.3 ± 0.3
	F	5	34.2 ± 4.2	1.4 ± 0.1
1% iodized casein	M	7	32.0 ± 0.7	1.5 ± 0.2
	F	3	28.5 ± 2.4	1.4 ± 0.2
0.04% propylthiouracil	M	5	35.6 ± 4.2	1.5 ± 0.3
	F	5	31.8 ± 1.7	1.5 ± 0.2
	With 10 μg vitamin B_{12}/kg diet			
None	M	5	27.2 ± 2.6	0.8 ± 0.2
	F	5	27.6 ± 1.2	0.7 ± 0.5
1% thyroid powder	M	5	26.8 ± 3.6	0.6 ± 0.2
	F	3	26.8 ± 3.3	0.8 ± 0.1
1% iodized casein	M	5	26.4 ± 1.7	0.8 ± 0.1
	F	5	26.2 ± 1.5	0.7 ± 0.2
0.04% propylthiouracil	M	6	27.8 ± 1.9	0.7 ± 0.1
	F	5	26.8 ± 1.8	0.8 ± 0.1

[1] ± SD.

duction in both serum and liver glutathione content 5 weeks after being transferred. Two animals fed the basal diet had serum levels of 20.5 and 20.0 mg/100 ml and two from the group supplemented with propylthiouracil, 22.0 and 23.0 mg/100. These values were even lower than those for the animals that had been fed the vitamin B_{12}-supplemented diets for the duration of the study, as shown in table 3. The liver glutathione content for these 4 animals was 0.8, 0.6, 0.6, and 1.2 mg/g.

Liver coenzyme A. The concentration of coenzyme A in the liver is shown in table 4. While there was an apparent variation among the various groups, no statistically significant differences were found either between the sexes, within the groups with and without vitamin B_{12}, or between parallel groups.

DISCUSSION

The urinary excretion of MMA and FIGlu by the vitamin B_{12}-deficient hamsters in this study, as well as the increased serum and liver glutathione levels, showed that the hamster requires a dietary source of this vitamin. That gain in body weight may not be a suitable criterion of need of this vitamin under these conditions is indicated by the fact that the animals continued to gain in weight similarly to the control animals. This similar gain in weight was observed even while MMA, which requires the vitamin B_{12} coenzyme for its further conversion to succinate (23), was being excreted by the vitamin B_{12}-deficient hamsters.

FIGlu may be excreted by other species deficient in either folic acid or vitamin B_{12} (8, 10). As ample folic acid was included in the diet and as the excretion of FIGlu ceased 5 weeks after the deficient animals had been fed the vitamin B_{12}-supplemented diets, it can be concluded that excretion of this compound was due in this case to deficiency of vitamin B_{12}.

Excretion of AIC has been reported also in deficiencies of either vitamin B_{12} or folic acid in both humans (9) and animals (10), but there was no increase in its excretion by the vitamin B_{12}-deficient hamsters in this study. McGeer et al. (10) in a study on AIC excretion in both folic acid and vitamin B_{12} deficiency, suggested that the existing pool of folic acid is used first for the breakdown of AIC. Such a suggestion is not inconsistent with the results of this study, as vitamin B_{12} deficiency may result in a functional folic acid deficiency

TABLE 4

Liver coenzyme A

Supplement to basal diet	Sex	No. rats	Coenzyme A
			units/g
Without vitamin B_{12}			
None	M	5	55 ± 8 [1]
	F	4	45 ± 25
1% thyroid powder	M	5	44 ± 7
	F	5	52 ± 13
1% iodized casein	M	7	56 ± 4
	F	3	53 ± 11
0.04% propylthiouracil	M	5	52 ± 5
	F	5	46 ± 6
With 10 μg vitamin B_{12}/kg diet			
None	M	5	53 ± 9
	F	5	46 ± 14
1% thyroid powder	M	5	46 ± 11
	F	3	44 ± 7
1% iodized casein	M	5	46 ± 10
	F	5	45 ± 11
0.04% propylthiouracil	M	6	45 ± 9
	F	5	42 ± 5

[1] ± SD.

(10). Since there was a span of 7 to 8 weeks between the excretion of MMA and FIGlu, perhaps the deficient hamsters would have excreted increased amounts of AIC had they been fed the deficient diets for a longer period of time.

That a vitamin B_{12} deficiency existed in the hamsters in the study reported here was indicated by the urinary excretion of MMA and FIGlu and increased serum and liver glutathione levels. According to the results of this study, the metabolism of glutathione and coenzyme A differ in the hamster from that in the rat, in that there was no change in liver coenzyme A levels and the serum and liver glutathione levels were increased rather than decreased in the deficient animals. O'Dell et al. (24) in a study of oxidized and reduced glutathione concentrations in vitamin B_{12}-deficient rats concluded that vitamin B_{12} is concerned with the maintenance of sulfhydryl compounds in the reduced state. This observation was confirmed by Biswas and Johnson (25), who reported that glutathione reductase activity was lowered in vitamin B_{12}-deficient chicks. No explanation is offered here for the observed difference in response of the hamster to vitamin B_{12} deficiency, but further studies on this deficiency in the hamster are in progress. Among the first determinations being made are the activity of glutathione reductase and vitamin B_{12} levels in various tissues.

The addition of the thyroid-regulating compounds to the diets of the hamsters did not appear to affect the development of the deficiency under these conditions. The only explanation is that thyroid metabolism may differ in the hamster, which is a hibernating animal, but it is not possible to describe those differences from the observations of the present study.

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Effect of Progressive Starvation on Rat Liver Enzyme Activities [1]

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ABSTRACT The patterns of decreases in total liver enzyme activities, body weight and some selected liver constituents in young and old rats were studied during progressive starvation. The following were observed in young rats: total liver protein, liver glycogen, phosphoglucomutase, fructose diphoshatase, aldolase, succinic dehydrogenase and fumarase decreased in activity after one day of starvation and remained relatively constant during the next 3 days of starvation. A decrease on days 1 and 4 of starvation was noted with soluble liver protein, phosphohexoseisomerase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase, α-glycerolphosphate dehydrogenase, malic enzyme (TPN), isocitric dehydrogenase (TPN) and malic dehydrogenase. A decrease in the activities of phosphorylase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and the pentose phosphate-metabolizing enzymes was observed on days 1, 2 and 4 of starvation. Glucose 6-phosphatase and uridine diphosphate glucose dehydrogenase activities remained relatively constant during 3 days of starvation and decreased on day 4. The patterns of decreases in total liver enzyme activities in old rats were similar to those for young rats with a few notable exceptions. The most striking of these was the sharp decrease in the activity of uridine diphosphate glucose dehydrogenase on day 1 of starvation in old rats. The activities of glucose 6-phosphatase and lactate dehydrogenase were maintained to a greater extent in young rats than in old rats. But the activities of aldolase, α-glycerolphosphate dehydrogenase, isocitrate dehydrogenase (TPN) and glucose 6-phosphate dehydrogenase appeared to be more resistant to decreases during starvation in old rats. In general, the activities of enzymes involved in gluconeogenesis, the citric acid cycle, and the pentose phosphate-metabolizing enzymes were equally stable to starvation. The activities of enzymes of the Embden-Meyerhoff pathway (phosphoglucomutase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and lactic dehydrogenase) were somewhat less stable. The activities of those enzymes which are thought to be associated with lipogenesis (glucose 6-phosphate dehydrogenase, malic enzyme and α-glycerolphosphate dehydrogenase) were the least stable. The possible physiological importance of this order of stability is discussed.

Numerous studies have been made of the effect of starvation on liver enzyme activity. In many studies, however, livers were examined after only a single starvation period, or only a few enzymes were examined. A report of 12 liver enzymes after a 7-day starvation showed that the method of reporting results is important to interpreting data (1). Six enzymes had an increased activity per gram of fresh liver weight or per milligram of liver protein. Four increased in terms of final body weight. All enzymes decreased in terms of initial body weight, and eleven decreased per liver nucleus. Considerable attention has been given to the effect of starvation upon the activities of glucose 6-phosphatase (2), phosphorylase (3), urea cycle enzymes (4), and TPN-linked enzymes (5, 6). Therefore, it appeared important to investigate the activities of many liver enzymes over various periods of starvation to determine whether metabolic function and maintenance of activity during starvation are related.

METHODS

All rats were fed a high glucose diet (7) for 5 days prior to starvation. During the starvation period, the animals were housed individually in screen-bottom cages and allowed water ad libitum.

The animals were killed by a sharp blow on the head, decapitated, exsanguinated,

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and the liver was removed rapidly and completely, and was weighed and chilled. Portions of the liver were treated as follows: preparation A, 100 to 200 mg were used for the determination of glycogen (8); preparation B, homogenized in 99 volumes of 0.1 M potassium phosphate, pH 7.4, for the succinic dehydrogenase assay (9); preparation C, in 19 volumes of 0.1 M potassium citrate, pH 6.5; and preparation D, in 4 volumes of 0.14 M KCl. Preparation D was centrifuged at 0–4° for 30 minutes at 20,000 × g, and the resulting supernatant solution was used as the enzyme source.

Two assays were conducted at 37° by the following methods: glucose 6-phosphatase for 15 minutes by a previously described method (10), and phosphorylase for 10 minutes (final concentrations in an 0.8-ml volume were 0.0375 M citrate, pH 6.5, 0.057 M KF, 0.0125 M glucose 1-phosphate, 6.3 times 10^{-6} M AMP, 0.25% glycogen, and enzyme). Both assays were stopped with 10% TCA, and inorganic phosphate determined (11).

The remaining assays were conducted at 25° by observing changes in absorbency with a Gilford Model 2000 Multiple Absorbence Recorder. All reactions containing TPN, DPN, or DPNH were followed by observing changes in absorbance at 340 mμ. Succinic dehydrogenase (9) and pentose phosphate-metabolizing enzymes (7) were determined by methods described elsewhere.

The following concentrations were used for assays not previously decribed in detail. The first six were TPN/TPNH reactions and the second six were DPN/DPNH reactions.

1. Phosphoglucomutase: 0.017 M glycylglycine, pH 7.6, 3.5×10^{-3} M $MgSO_4$, 0.33×10^{-3} M TPN, 0.67×10^{-3} M glucose 1-phosphate, glucose 6-phosphate dehydrogenase 0.03 units/ml, and enzyme.
2. Phosphohexose isomerase was similar to phosphoglucomutase except that 0.33×10^{-2} M fructose 6-phosphate was added to the reaction mixture in place of the glucose 1-phosphate.
3. Glucose 6-phosphate dehydrogenase was similar to phosphoglucomutase except that no glucose 6-phosphate dehydrogenase was added, and 0.67×10^{-3} M glucose 6-phosphate was added in place of glucose 1-phosphate.
4. 6-Phosphogluconate dehydrogenase assay was similar to that for glucose 6-phosphate dehydrogenase except that 0.67×10^{-3} M 6-phosphogluconate was used in place of glucose 6-phosphate.
5. Isocitrate dehydrogenase: 0.017 M glycylglycine, pH 7.6, 7×10^{-3} M $MgSO_4$, 0.7×10^{-3} M $MnSO_4$, 0.33×10^{-3} M TPN, 0.33×10^{-2} M isocitrate, and enzyme.
6. Malic enzyme assay was similar to the isocitric dehydrogenase assay except that 0.33×10^{-2} M malate was added in place of isocitrate.
7. Glyceraldehyde 3-phosphate dehydrogenase: 1.7×10^{-2} M arsenate and 2.7×10^{-2} M glycine, both at pH 9.0, 2.0×10^{-2} M cysteine, pH 9.0, 4.5×10^{-3} M DPN, 3.5×10^{-3} M $MgSO_4$, 6.7×10^{-3} M fructose 1,6-diphosphate, 0.6 units/ml of aldolase, and enzyme.
8. Aldolase assay was similar to that for glyceroaldehyde 3-phosphate dehydrogenase except that 2.2 units/ml of glyceroaldehyde 3-phosphate were added in place of the aldolase.
9. Lactate dehydrogenase: 0.033 M tris, pH 7.4, 0.22×10^{-3} M DPNH, 3.3×10^{-4} M pyruvate, and enzyme.
10. L-α-Glycerolphosphate dehydrogenase: 0.033 M tris, pH 7.4, 0.22×10^{-3} M DPNH, 6.7×10^{-3} M fructose 1,6-diphosphate, 0.6 units/ml of aldolase, and enzyme.
11. Uridine diphosphate glucose dehydrogenase: 0.1 M glycine, pH 8.7, 0.83×10^{-3} M DPN, 5.0×10^{-4} M UDPG, and enzyme.
12. Malate dehydrogenase assay was similar to that for lactate dehydrogenase except that 2.5×10^{-4} M oxalacetate (freshly prepared) was added in place of the pyruvate.
13. Fumarase: by measuring the change in absorbance at 240 mμ; 0.033 M tris, pH 7.4, 8.3×10^{-4} M fumarate, and enzyme.

The following preparations were used for the accompanying assays after appro-

priate dilutions: preparation B for succinic dehydrogenase; preparation C for glucose 6-phosphatase and phosphorylase; preparation D for phosphoglucomutase, phosphohexoseisomerase, aldolase, glyceraldehyde 3-phosphate dehydrogenase, L-α-glycerolphosphate dehydrogenase, lactate dehydrogenase, malic dehydrogenase, isocitric dehydrogenase, malic enzyme, fumarase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, uridine diphosphateglucose dehydrogenase, and protein (12).

RESULTS

The data are presented in such a way that the activities can be recalculated on the basis of activity per unit of fresh liver weight, per gram of liver protein and per unit of final body weight. Since it was of interest to examine changes in total enzyme activity, the emphasis was put upon reporting enzyme activity per unit of original body weight. This method of reporting enzyme activity was decided upon because the decrease in body weight, liver size and liver protein during starvation would tend to obscure a decrease in enzyme activity. For example, if the total activity of an enzyme decreased by 30% during starvation and liver protein decreased by 50%, the activity of the enzyme reported as units per milligram of liver protein would appear to have increased, while in actuality the total activity of the enzyme decreased. This would also be true of changes using other bases of reporting the results. For further ease of comparison, all values subsequent to the beginning of starvation are reported as a percentage of the fed values.

The first experimental group contained rats with starting weights of approximately 130 g. Their body weight shows a continuous decrease with period of starving (table 1). The ratio of liver weight to body weight was markedly decreased after 24-hours of starvation, remained constant for the next 2 days, then decreased again. Liver protein decreased after the first day of starva-

TABLE 1

Effect of starvation on several liver constituents in young rats [1]

	Before starvation	Starvation period in days			
		1	2	3	4
		% of pre-starvation values			
Body weight, g	130	89±1	86±2	76±2	75±2
Liver weight, g/100 g body wt	5.47	64±3	65±4	67±1	51±2
Liver protein, mg/100 g body wt (total)	753±71 [2]	75±2	87±7	75±7	72±4
Liver protein, mg/100 g body wt (soluble)	591±28	64±2	69±4	75±2	60±5
Liver glycogen, mg/100 g body wt	238±29	1±1	6±1	6±2	9±3
Glucose 6-phosphatase	90.6 ±10.7	101±7	113±11	95±15	66±4
Phosphoglucomutase	14.3 ± 0.5	49±2	52±2	48±1	40±10
Aldolase	9.28± 0.72	58±8	61±3	51±5	57±5
Phosphohexoseisomerase	549±51	77±4	77±3	68±3	44±5
Fructose 1,6-diphosphatase	11.3 ± 0.7	65±4	56±3	69±2	51±4
Glyceraldehyde 3-phosphate dehydrogenase	380±38	52±3	51±6	45±0	32±3
Lactate dehydrogenase	2058±142	83±5	84±14	77±4	50±4
Phosphorylase	65.4 ± 9.0	77±5	54±7	52±7	30±3

[1] Values in first column, except for the first 5 items, are given as micromoles of substrate converted per 100 g body weight/minute. Values in the subsequent columns are as a per cent of the pre-starvation values, i.e., for absolute values multiply the first column by the per cent on the desired day of starvation. This will give the value per original 100 g of body weight.

[2] SE of mean. All values are the average of 4 animals.

tion and then remained constant. Liver glycogen decreased after one day and remained low. The effect of starvation on the activities of various liver enzyems concerned with glycolysis and gluconeogenesis appear to fall into several patterns (table 1). The activity of one enzyme, glucose 6-phosphatase, was maintained for 3 days and then declined sharply. The activities of 2 enzymes, phosphoglucomutase and aldolase, decreased markedly after one day of starvation and then remained constant throughout the remainder of the experimental period. Four enzymes, phosphohexoseisomerase, fructose 1,6-diphosphatase, glyceraldehyde 3-phosphate dehydrogenase, and lactate dehydrogenase, decreased in activity after one day of starvation, but to varying extents. These enzymes remained at a constant level of activity for the next 2 days and then decreased again. Phosphorylase activity also decreased during starvation, but the pattern of decline was somewhat different from that of the other 7 enzymes.

Enzymes that have been indirectly associated with lipogenesis, the pentose shunt enzymes, and citric acid cycle enzymes were also studied (table 2). The activities of the pentose-shunt enzymes, (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and pentose phosphate-metabolizing enzymes) all decreased after the first day and then decreased further on the second and fourth days of starvation. The malic enzyme activity decreased markedly on the first day, remained constant until the fourth day, and then decreased further. L-α-Glycerolphosphate dehydrogenase also decreased very markedly one day after fasting, remained at this level on the following day and then showed noticeable decreases in activity on the following 2 days. Uridine diphosphate glucose dehydrogenase showed slight decreases on the first day and remained fairly constant over the next 2 days, showing a very marked decrease on the fourth day after starvation. Most enzymes of the citric acid cycle decreased after the first day of starvation, remained relatively constant for the next 2 days, and showed a slight further decrease on the fourth day.

Trends with the heavier rats (table 3) were similar to trends with the lighter rats. There were certain exceptions, however. Among these were a more severe decrease in lactate dehydrogenase and glucose 6-

TABLE 2

Effect of starvation on several liver enzymes in young rats [1]

	Before starvation	Starvation period in days			
		1	2	3	4
		% of pre-starvation values			
Glucose 6-phosphate dehydrogenase	71.3 ± 12.3 [2]	44 ± 8	23 ± 3	34 ± 4	11 ± 2
6-Phosphogluconate dehydrogenase	3.50 ± 0.10	64 ± 4	44 ± 6	46 ± 4	27 ± 3
Pentose phosphate-metabolizing enzymes	5.64 ± 0.31	79 ± 2	70 ± 2	66 ± 2	54 ± 5
Malic enzyme	35.2 ± 5.2	36 ± 3	37 ± 2	35 ± 5	16 ± 2
α-Glycerol phosphate dehydrogenase	342 ± 7	50 ± 5	54 ± 4	44 ± 1	33 ± 4
Uridine diphosphate glucose dehydrogenase	0.405 ± 0.123	89 ± 2	97 ± 9	89 ± 7	39 ± 7
Isocitric dehydrogenase (TPN)	99.2 ± 3.8	69 ± 3	78 ± 11	61 ± 1	50 ± 5
Succinic dehydrogenase	14.9 ± 1.2	70 ± 6	79 ± 10	75 ± 4	67 ± 9
Fumarase	39.4 ± 3.6	71 ± 4	79 ± 14	75 ± 4	67 ± 16
Malic dehydrogenase	1980 ± 71	84 ± 4	87 ± 8	84 ± 5	68 ± 5

[1] Values in first column are given as micromoles of substrate converted per 100 g body weight/minute. Values in the subsequent columns are as a per cent of the pre-starvation values, i.e., for absolute values multiply the first column by the per cent on the desired day of starvation. This will give the value per original 100 g of body weight.

[2] SE of mean. All values are the average of 4 animals.

phosphatase, and a later decrease in fructose diphosphatase and aldolase activities. It should also be noted that among rats fed ad libitum the heavier rats had a higher activity of phosphorylase and the lighter rats had higher activities of glucose 6-phosphatase, fructose diphosphatase, aldolase, and lactate dehydrogenase. In heavier rats the pentose phosphate pathway dehydrogenase enzyme activities decreased markedly in the first 2 days (table 4), and then decreased somewhat more in the next 2 days. In contrast with this, the pentose phosphate-metabolizing enzyme activity in the heavy rats did not decrease until the second day and then again on the third day, but no decrease was noted on the fourth day. The activities of α-glycerolphosphate dehydrogenase, malic enzyme, isocitrate dehydrogenase and malic dehydrogenase in the heavier rats showed patterns similar to those in the lighter rats,

TABLE 3

Effect of starvation on several liver constituents in older rats [1]

	Before starvation	Starvation period in days			
		1	2	3	4
		% of pre-starvation values			
Body weight, g	344	92±1	89±1	89±1	82±1
Liver weight, g/100 g	4.21	58±2	56±3	51±3	44±3
Liver protein, mg/100 g (total)	1156± 96 [2]	82±1	84±3	62±2	68±2
Liver protein, mg/100 g (soluble)	581± 20	89±9	69±5	55±4	65±3
Liver glycogen, mg/100 g	198± 17	2±1	4±1	5±1	7±1
Phosphorylase	82.2 ± 7.5	61±4	70±2	50±5	39±2
Phosphoglucomutase	12.0 ± 1.4	65±5	55±2	40±3	47±6
Lactate dehydrogenase	1282±105	66±6	65±10	59±2	57±3
Glucose 6-phosphatase	62.5 ± 2.7	105±19	86±10	67±4	68±12
Fructose diphosphatase	8.44± 0.74	89±4	64±4	60±6	54±4
Aldolase	3.97	100±40	63±23	78±34	59±23

[1] Values in first column, except for the first 5 items, are given as micromoles of substrate converted per 100 g body weight/minute. Values in the subsequent columns are as a per cent of the pre-starvation values, i.e., for absolute values multiply the first column by the per cent on the desired day of starvation. This will give the value per original 100 g of body weight.
[2] SE of mean. All values are the average of 4 animals.

TABLE 4

Effect of starvation on several liver enzymes in older rats [1]

	Before starvation	Starvation period in days			
		1	2	3	4
		% of pre-starvation values			
Glucose 6-phosphate dehydrogenase	30.6 ±5.8 [2]	70±5	46±5	39±3	30±3
6-Phosphogluconate dehydrogenase	2.23±0.21	56±5	40±4	32±2	25±1
Pentose phosphate-metabolizing enzymes	3.81±0.37	97±5	74±1	62±2	63±3
Malic enzyme	13.6 ±1.2	50±5	39±6	31±3	25±1
Isocitric dehydrogenase (TPN)	67.8 ±2.6	75±4	68±1	65±4	62±1
Fumarase	32.9 ±2.6	88±5	94±7	54±3	65±9
Malic dehydrogenase	464±47	91±33	75±11	65±13	76±3
Uridine diphosphate glucose dehydrogenase	2.97±0.36	18±5	29±3	8±0	21±5
α-Glycerol phosphate dehydrogenase	261±13	65±4	58±5	37±2	48±4

[1] Values in first column are given as micromoles of substrate converted per 100 g body weight/minute. Values in the subsequent columns are as a per cent of the pre-starvation values, i.e., for absolute values multiply the first column by the per cent on the desired day of starvation. This will give the value per original 100 g of body weight.
[2] SE of mean. All values are the average of 4 animals.

with some minor differences in the pattern of decrease. The fumarase activity remained high for 2 days, decreased somewhat on the third day, but even after 4 days was not greatly reduced. The UDPG dehydrogenase activity dropped to a low level after the first day and remained there. This is in strong contrast with results obtained with the lighter rats.

DISCUSSION

Aldolase, fructose 1,6-diphosphatase, glucose 6-phosphate dehydrogenase, and pentose phosphate-metabolizing enzyme activities appeared to be slightly more stable to starvation in the heavier rats, decreasing about one day later than in lighter rats. In contrast, lactate dehydrogenase and uridine diphosphate glucose dehydrogenase were more stable in the lighter rats. Most of the other enzymes show comparable behavior in rats of both weights. The reason for this difference due to period of starvation is not clear.

A very large number of enzyme activities decreased significantly after one day of starvation, showed only slight or no decrease in the following 2 days, and decreased significantly further on the fourth day. It is not certain why the systems behave in this fashion, but several possibilities exist. The first possibility is a large scale change in the internal nutrition of the rats on the first day and from the third to fourth days of starvation. The second possibility is the stability of the template or messenger RNA, which may decay without replenishment between the third and fourth days in some of these systems, as there is evidence that certain messenger RNA's may have a life-span rather than a half-life (13). There is also a possibility of a change in hormone balance from the third to fourth day, for it has been shown that certain enzymes in starved rats decrease more rapidly in rats with various endocrine removals than in normal rats (14). And again, the stability, or turnover time, of certain enzymes may be altered significantly during starvation as has been shown for arginase (15). These are only several of the many possible explanations.

These experiments indicate that the stability during starvation of various liver constituents and enzyme activities is not uniform. The observed changes may reflect the relative importance of each system during this period, or may reflect changes associated with changing sources of substrates during starvation. The enzymes that are relatively more stable under these circumstances are those associated with gluconeogenesis (glucose 6-phophatase and fructose diphosphatase, although the latter appears less stable), glycolysis (phosphoglucomutase, aldolase, glyceraldehyde 3-phosphate dehydrogenase and lactic dehydrogenase) and citric acid cycle enzymes. The first 2 processes are of great importance to the animal, especially in the absence of dietary glucose, and the third is of prime importance to the cell itself, for energy production. This preferential maintenance was observed with both the heavier and lighter rats. The activities of enzymes thought to be indirectly involved in lipogenesis (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and α-glycerolphosphate dehydrogenase) were the least stable. This may reflect the absence of lipogenesis during starvation; therefore, the enzymes associated with lipogenesis are dispensible in such a situation.

Finally, it should be kept in mind that starvation necessitates the activation in situ of complicated control mechanisms in order to conserve those components of the cell which are essential to the maintenance of life. Less essential components can be utilized as the source of building blocks for the maintenance of essential components as well as for the production of energy. This argues for the preferential maintenance of the activities of some enzymes at the expense of others. However, since the existence of complicated control mechanisms is implied, activities in vivo may differ substantially from activities in vitro. This necessitates a great deal of caution in interpreting data obtained with measurements in vitro. Nevertheless, optimal enzyme activities, as measured in vitro, can provide us with some insight into the physiological situation and stimulate further experimental inquiry.

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Oxalate Metabolism in the Pack Rat, Sand Rat, Hamster, and White Rat [1]

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ABSTRACT Soluble oxalates are toxic because they bind calcium in the organism and are not metabolized, but certain rodents appear to be able to tolerate a considerable amount of soluble oxalates and to absorb calcium from insoluble calcium oxalate. The metabolism of oxalates by pack rats (*Neotoma albigula*), hamsters (*Mesocricetus auratus*), sand rats (*Psammomys obesus*) and Osborne-Mendel white rats (*Rattus norvegicus*) was investigated by (a) feeding insoluble calcium oxalate and studying the degradation of the oxalate and the excretion of calcium in the urine; (b) feeding ^{14}C-labeled soluble oxalate and observing its metabolism; and (c) injecting ^{14}C-oxalate and following its elimination from the body. When calcium oxalate was fed, some of the oxalate was degraded and some of the calcium appeared in the urine of pack rats, hamsters, and sand rats, but white rats did not metabolize oxalate or increase calcium excretion. When soluble oxalate was ingested, three of the rodents degraded almost all of it, but white rats degraded only half. Injected oxalate was excreted in the urine by pack rats and hamsters, some sand rats excreted oxalate and others degraded it, and white rats eliminated half in the urine and half in the feces. These observations suggest that oxalate degradation takes place in the intestine, presumably by microbial action.

Oxalic acid and soluble oxalates are toxic, primarily because they bind calcium in the organism and are not metabolized or degraded. The insoluble calcium oxalate, however, is physiologically inert; its calcium is not ordinarily available and the oxalate remains unabsorbed. Yet, amounts of soluble oxalate in proportion to the body weight that would be lethal if given to man appear to be harmless to sand rats (*Psammomys obesus*). It has been suggested that other rodents as well may have a different oxalate metabolism (1). Pack rats (*Neotoma albigula*) appear to degrade the oxalate in calcium oxalate, for they absorb calcium from this insoluble compound. When they are fed calcium oxalate their urine appears milky due to a precipitate of calcium carbonate, yet when white rats ingest Ca oxalate, their urine remains clear, as its calcium content does not increase. The urine of hamsters is often thick and creamy, which suggests that they may have a calcium and oxalate metabolism similar to that of the pack rat. We examined the absorption of calcium from calcium oxalate, as well as the metabolism of soluble oxalate by pack rats, hamsters, sand rats, and white rats.

The extent to which these rodents excreted calcium from dietary Ca oxalate and tolerated soluble oxalate in their diet was studied in a series of 4 experiments. (a) The rodents were fed calcium oxalate to learn whether they absorbed and excreted calcium from this compound. (b) They were fed calcium oxalate-^{14}C to determine whether the calcium absorption observed in (a) was accompanied by oxalate degradation. (c) They were fed sodium oxalate-^{14}C to learn whether soluble oxalate would be metabolized to a greater extent than insoluble oxalate. (d) Oxalic acid-^{14}C was injected to determine whether the oxalate degradation observed in (b) and (c) could have taken place outside the gastrointestinal tract.

MATERIALS AND METHODS

Animals. The sand rats, *Psammomys obesus* Cretzschmar (100 to 240 g) were captured in Egypt and were maintained

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with fresh beets supplemented by carrots and spinach. The pack rats, *Neotoma albigula* Hartley, (120 to 260 g) came from the Santa Rita Experimental Range in Arizona. These, the Syrian hamsters, *Mesocricetus auratus*, (75 to 200 g) and the Osborne-Mendel white rats, *Rattus norvegicus*, (250 to 500 g) were fed commercial laboratory ration,[3] lettuce, and water.

Feed and oxalate administration. When oxalates were to be given orally, they were incorporated into the diet, which during the feeding experiments was a baked whole wheat yeast bread. The bread was made from 7.5 g dried yeast,[4] 7.5 g sugar, 275 g whole wheat flour, 200 ml water, and the oxalate. This was baked at 180°, and dried to constant weight before use. For calcium oxalate feeding, 25 g Ca oxalate were sifted into the dough. For a uniformly labeled Ca-oxalate-^{14}C feed a low specific activity calcium oxalate-^{14}C was made by adding 26 g $CaCl_2 \cdot H_2O$ to a solution of 24.3 g $(NH_4)_2C_2O_4 \cdot H_2O$, and 50 μCi oxalic acid-1,2-^{14}C (Volk). The pH of the solution was adjusted to pH 4 with concentrated NH_4OH, and after standing overnight the precipitate was centrifuged and washed twice with water. For sodium oxalate-^{14}C bread 12.5 g sodium oxalate and 50 μCi oxalic acid-1,2-^{14}C were dissolved in the water. The feed was homogeneous as different samples from the same batch had the same calcium or ^{14}C content. At least 3 animals from each species were used in these experiments. The feed was given ad libitum: intakes ranged from 5 to 20 g/day, with a calcium oxalate content of 0.4 to 1.7 g or a sodium oxalate content of 0.2 to 0.9 g.

For administration of oxalate by injection a solution was prepared by dissolving 50 μCi (1.5 mg) oxalic-1,2-^{14}C-acid (Volk) in 5 ml of 0.9% NaCl. Just before use this solution was diluted 1:10 with 0.9% NaCl giving a final concentration of 1 μCi/ml fluid and 2 ml solution were injected intraperitoneally. During the injection experiments the animals were fed nonradioactive Ca oxalate.

Metabolic cages. Each animal was kept in a wire-floor cage (24 × 18 × 18 cm). The cage was placed above an incline covered by Whatman no. 3MM chromatography paper. As the excreta fell on the incline, the urine was absorbed by the paper (or dripped off the paper into a narrow plastic trough placed at its lower edge), and all fecal pellets rolled off the paper and into a pan beyond the trough.

In experiments in which CO_2 was collected, the above assembly was put into a glass box (64 × 24 × 56 cm). Room air was pulled through the box and through two CO_2 traps containing 150 ml 5 N NaOH and 150 ml 1 N NaOH. CO_2 entering the system was measured in a separate train of absorbers. The flow rate was adjusted with the aid of a flow meter at the beginning of each day to make it equal in all trains.

Chemical analyses. To extract calcium from the urine, those portions of the chromatography paper discolored by urine were cut out and extracted in 100 ml 1 N HCl for at least 3 hours. The calcium content of an equal area of clean paper was determined to correct for calcium present in the paper. Bread samples were pulverized and extracted in 100 ml 2 N HCl for 20 hours. Calcium determinations were made by complexing with EGTA and back-titrating with $CaCl_2$ using Calcon as an indicator (2).

The ^{14}C assay of samples of feed and feces were made on CO_2 collected after acid digestion with fuming sulfuric acid and chromium trioxide (3). Urine samples were treated with 0.4 N H_3PO_4 to release CO_2 from carbonates, and in the soluble oxalate experiments an aliquot of the acid was digested with the Van Slyke mixture. The CO_2 released by digestion was collected in 150 ml 0.1 N NaOH. A 12-ml sample of the sodium hydroxide containing labeled carbonates was mixed with 4.0 g anthracene and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 314 EX (4).

RESULTS

Calcium oxalate experiments. Oxalate degradation of ingested calcium oxalate and calcium absorption and excretion from this "insoluble" compound were tested in two feeding experiments. The amounts of calcium excreted in the urine and of oxalate degraded to CO_2, relative to the in-

[3] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[4] Fleischmann's, General Foods, New York.

gested amount, are shown in figure 1. The total quantities of calcium excreted in the urine per day by the rodents were as follows: (mg Ca/100 g body wt) pack rats, 15; hamsters, 6; sand rats, 0.2; and white rats, 0.8. The quantities of radioactivity from Ca oxalate-^{14}C recovered per day in the expired CO_2 were as follows: (count/min/100 g body wt) pack rats, 4600; hamsters, 1700; sand rats, 1000; and white rats, 15. The remainder of the ^{14}C was recovered in the feces; only trace amounts were noted in the urine. Therefore pack rats and hamsters absorbed and excreted in the urine some of the calcium and metabolized some of the oxalate in Ca oxalate. Sand rats metabolized some of the oxalate, but did not increase their urinary calcium excretion. White rats eliminated calcium oxalate in the feces.

Soluble oxalate experiments. The animals were fed sodium oxalate-^{14}C to determine whether ingested soluble oxalate could be metabolized. The white rats degraded somewhat less than one-half of the ingested oxalate, whereas the other 3 rodents degraded almost all (fig. 2). The mean ^{14}C count rates per day in the expired CO_2 were: (count/min/100 g body wt) pack rats, 4800; hamsters, 6000; sand rats, 5900; and white rats, 3400.

To determine whether oxidation of soluble oxalate took place before or after absorption from the intestinal tract, oxalic acid-^{14}C was injected into the animals. The results show that most of the activity was recovered in the urine (fig. 3). Pack rats excreted 6900, and hamsters 5900 count/min/100 g body weight in the urine. Three sand rats excreted most of the activity in

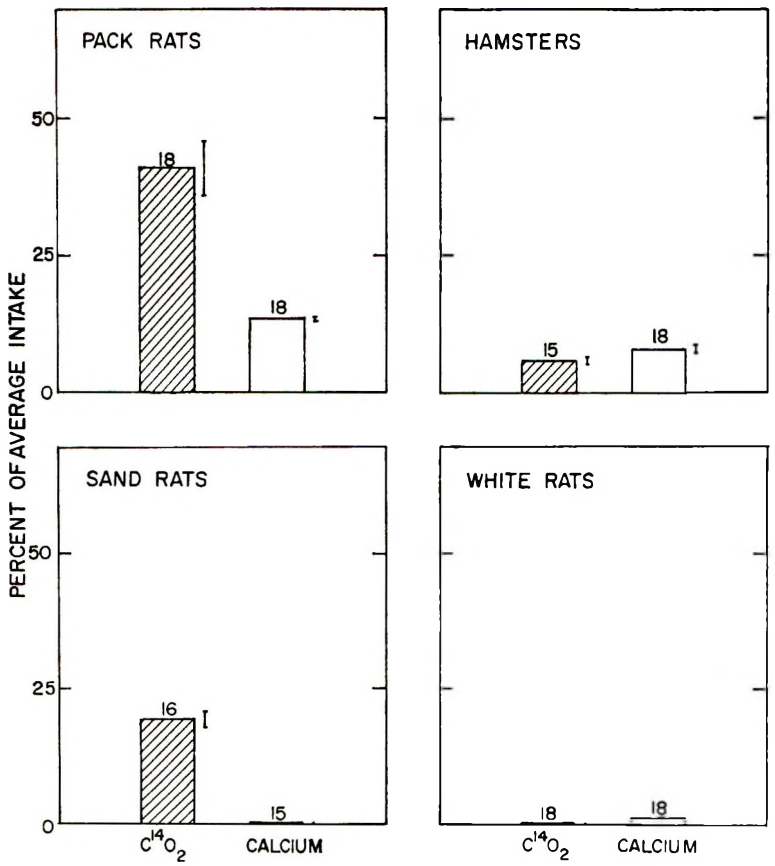

Fig. 1 Relative amounts of ^{14}C found in the expired CO_2 and of calcium excreted in the urine with a diet containing Ca oxalate-^{14}C. Numbers above bars give the number of one-day experimental periods used for estimate of standard errors. Vertical lines give ± SE.

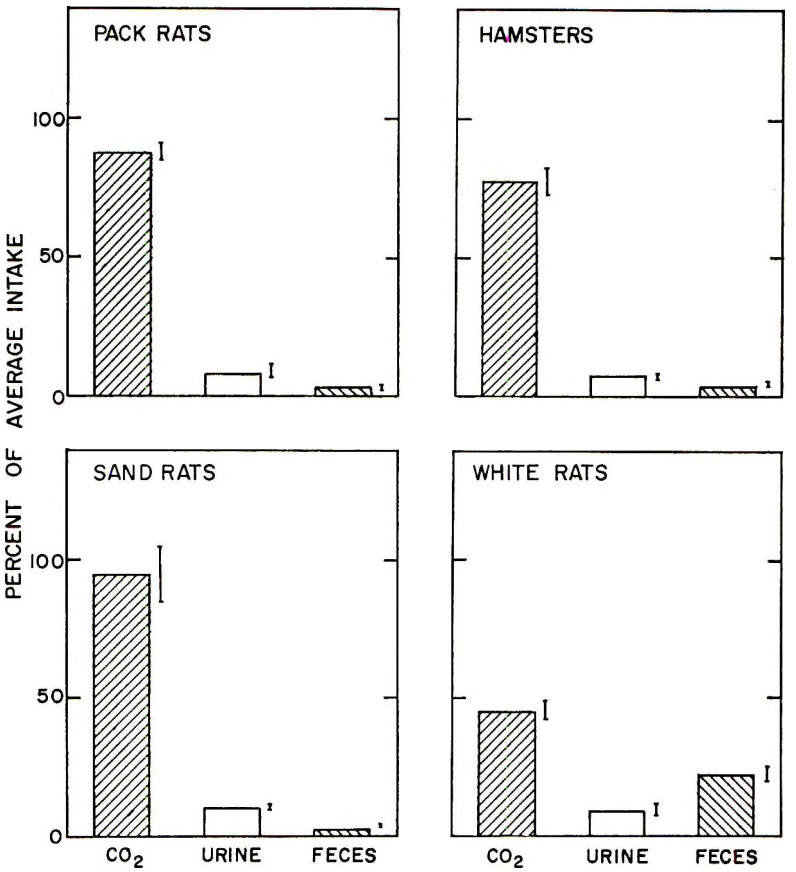

Fig. 2 Average recovery of ^{14}C from dietary sodium oxalate-^{14}C relative to the intake. Each bar represents the average of 3 animals studied for 6 days ± SE.

the urine, 8300 count/min/100 g body weight, and only 1400 in the respiratory CO_2; but two excreted 2600 in the urine against 9500 in the respiratory CO_2. The reason for this difference remains unknown. Although the white rats showed more radioactivity, 3400, in the feces than the other rodents, they still excreted 3000 count/min/100 g body weight in the urine. Thus the urine was a major route of excretion of injected oxalic acid-^{14}C in most of the rodents.

DISCUSSION

Calcium absorption. Pack rats and hamsters have an unusual ability to absorb and excrete calcium from Ca oxalate. There are several possible explanations. (i) Perhaps the calcium-absorbing mechanism can absorb Ca ions from the extremely low concentrations of calcium provided by the slight dissociation of Ca oxalate (sol. prod. = 2.57×10^{-9} M). That absorption of calcium from such low concentrations is possible was shown by Hasselbach and Makinose (5) who observed calcium uptake by muscle relaxing factor granules from a medium containing 5 mM oxalate, but only 0.1 mM Ca. (ii) Oxalate degradation may utilize the small quantities of oxalate in solution, leaving the calcium for absorption. This is consistent with the observation that more oxalate was metabolized than calcium absorbed and excreted by pack rats and sand rats. (iii) The absorption of calcium from Ca oxalate could also be aided by a complexing substance rendering Ca oxalate soluble. (iv) Ca oxalate could be absorbed by intestinal phagocytosis, but if this were true, then

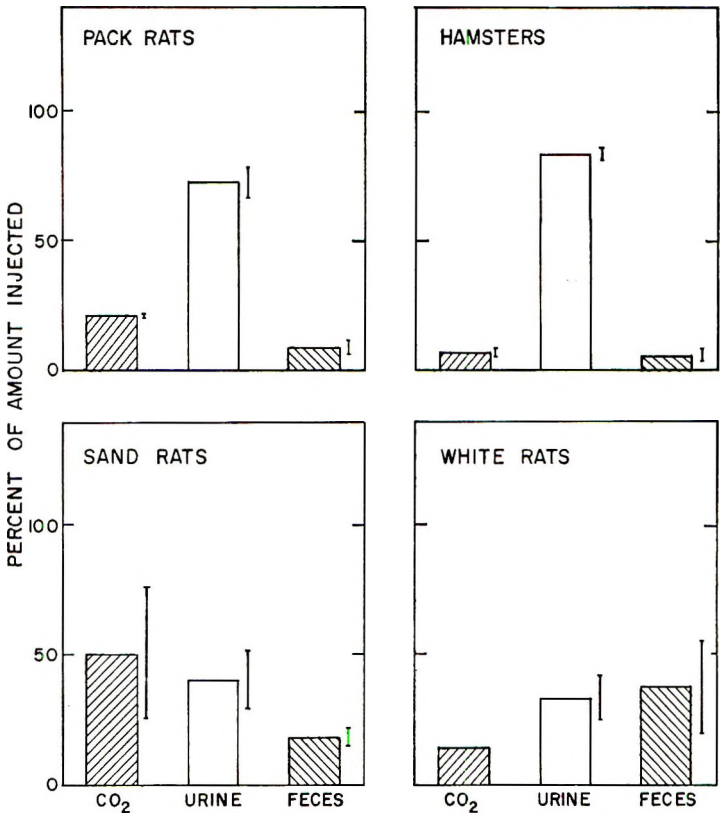

Fig. 3 Average recovery of ^{14}C from injected oxalic acid-^{14}C expressed as percentage of the amount injected. The bars represent an average of 3 animals (five for sand rats) ± SE.

radioactivity could be expected to appear in the urine, rather than in the CO_2. It appears likely that the calcium absorption resulted from a combination of calcium-absorbing and oxalate-metabolizing mechanisms working on low concentrations of substrate in the intestine.

The ability of white rats to utilize calcium in Ca oxalate has been studied by Causeret and Hugot (6). They fed young (70–100 g) rats a low calcium diet supplemented with various amounts of Ca oxalate or Ca carbonate. Their animals were able to retain 23 to 69% of the calcium from Ca oxalate and 44 to 94% from Ca carbonate. The absorption of Ca carbonate was accompanied by a slight increase in urinary calcium with the Ca oxalate diet, but this increase was a small fraction of what it was for pack rats and hamsters. Although urinary calcium excretion does not represent the amount absorbed, it is unlikely that the white rats absorbed much calcium. All the radioactivity from Ca oxalate-^{14}C was recovered in the feces, and calcium oxalate probably passed through the intestinal tract as an inert substance. Perhaps white rats that are young and have been maintained with low calcium diets, such as the ones used by Causeret and Hugot, absorbed calcium from calcium oxalate more readily than our mature animals that had an adequate calcium intake up to the time of the experiment.

Oxalate metabolism. Dietary soluble oxalate was metabolized by the rodents; yet reports in the literature indicate that mammalian enzymes do not catalyze oxalate metabolism. Such reports are based on studies of vitamin B_6 deficiency (7), hyperoxaluria (8), feeding tests (9) and injection experiments. Weinhouse and Friedman (10) injected oxalic acid-^{14}C into white rats and recovered 80% of the in-

jected activity as oxalate in urine, feces, and carcass. Curtin and King (11) also recovered injected oxalic acid-^{14}C from rats as urinary and fecal oxalate.

In our oxalic acid-^{14}C injection experiment, most of the radioactivity was recovered in the urine. Since the activity was not present as carbonate, in all probability it represents oxalate excretion. Some of the injected soluble oxalate appeared as CO_2, but it is still possible that the oxalate entered the intestine and that the degradation was due to microbial action. The fact that some ^{14}C was found in the feces suggests that injected oxalate does cross the intestinal wall.

Evidence for intestinal metabolism of oxalates has come from studies on ruminants as well as from the present study on rodents. The rumen contents of cattle eating oxalate-rich diets can destroy soluble oxalates in vitro (12). *Oxalis cernua* with 8 to 14 g oxalic acid/100 g dry weight has poisoned sheep when they were first introduced to it, but they appeared to develop a tolerance to it. Sheep that can eat *Oxalis* with impunity are still poisoned if small amounts of sodium oxalate are dripped into the abomasum or blood stream. If an animal has been feeding on *Oxalis*, its rumen liquor can destroy soluble oxalate in vitro. Presumably microbes established in the rumen enable sheep to tolerate the ingestion of large quantities of oxalate (13). Although most microorganisms do not utilize oxalic acid, there are a few bacteria and molds which do (14). The slight activity that appeared in the CO_2 of white rats injected with oxalic acid-^{14}C could not be measured after the intestine was sterilized with chloromycetin-palmitate (15). Therefore the oxalate metabolism observed in our rodents could probably be the result of intestinal microorganisms.

Biological significance. The unusual ability of the pack rat to absorb calcium might be a means of adapting it to a low calcium, high oxalate diet. Major components of the diet of pack rats in Arizona are cactus and mesquite (16). Neither jumping cholla (*Opuntia fulgida*), prickly pear (*O. engelmanni*), mesquite leaves, nor mesquite beans contain much water-soluble oxalate, but they all contain more acid-soluble oxalate than spinach (unpublished data). If the calcium-absorbing mechanism is not an adaptation to the diet it may mean only that the pack rat lacks the ability to regulate its absorption of calcium.[5]

The sand rat normally eats large quantities of oxalate in its diet. *Traganum nudatum* and *Suaeda mollis*, which are part of its staple food in the wild, contain some 0.5 N oxalic acid in the sap (1). With such a diet sand rats might eat 500 mg oxalic acid a day. In the laboratory, sand rats can be maintained with 15 g beet leaves, 20 g beet roots, 20 g carrots, and 50 g spinach a day,[6] a diet containing about 300 mg soluble oxalate per day. Therefore the ability to metabolize oxalate is important for the sand rat.

Hamsters and white rats probably do not usually obtain large quantities of soluble oxalate in their diets, for they have been fed in the laboratory for generations. Yet with respect to calcium and oxalate metabolism, the hamster is more similar to the pack rat than to the white rat. Perhaps the differences between the hamster and white rat are based more on constitutional than on dietary factors. The white rat (which in its oxalate metabolism differed most from the other species) belongs to a different family (Muridae) from the other three rodents (Cricetidae). The pack rat and hamster (which showed the greatest similarity) are classified by Simpson (17) in the same subfamily (Cricetinae). Thus the differences observed in the calcium and oxalate metabolism of these species correlate with their taxonomic relationship. Therefore it appears that both taxonomic and dietary factors affect the ability to metabolize oxalate.

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Plasma Amino Acid Pattern of Chicks in Relation to Length of Feeding Period

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ABSTRACT The concentration of either lysine or valine was varied from deficient to excessive dietary levels in a basal diet containing crystalline amino acids in order to study the relationship between amino acid levels and the accumulation of free amino acids in the blood plasma of chicks. In separate assays where the feeding phase was restricted to 30 minutes, increasing the dietary concentration of valine or lysine resulted in the accumulation of these amino acids in the plasma even though the lower dietary amino acid levels were known to be well below the concentration needed to maximize weight gain. Plasma lysine also increased when the feeding period was lengthened to 6 hours. In contrast, valine did not start to accumulate in plasma until the dietary concentration approached or exceeded the level known to be required for maximal gain.

Zimmerman and Scott (1) reported data on the concentration of amino acids in the plasma of chicks that had been fed experimental diets containing graded levels of a single essential amino acid (lysine, valine or arginine) over a period of 7 days. Blood samples were taken for analysis at the end of the test period. The crystalline amino acid mixture used in the study was complete except for the amino acid under study. In no case did the amino acid accumulate in the plasma so long as it remained first-limiting for growth. Not until dietary excesses of an amino acid were fed was there a rapid increase of that amino acid in blood plasma. From the plasma pattern it was possible to predict, with a high degree of accuracy, the concentration of the amino acid in the diet required to maximize chick growth. In the present study, similar diets were used, but the feeding phase of the assay was reduced to either 6 hours or 30 minutes in order to determine whether the duration of the feeding period would alter the aforementioned plasma amino acid pattern.

EXPERIMENTAL

All procedures relating to the handling of chicks, diets used and preparation of plasma samples for analysis by ion exchange chromotography have been outlined in previous reports (1,2).[2] Amino acid mixture B [3] (1) was used in all experiments, except that in experiment 1 the mixture contained 12% L-glutamic acid. From the seventh to tenth day the chicks received an isolated soybean protein-glucose diet. After going through a fasting procedure on the tenth day the chicks were weighed and assigned at random to treatments on the eleventh day. From the eleventh to fourteenth day the chicks received the diets containing the complete amino acid mixture. On day 14 they were fasted for 4 hours prior to being offered the test diets.

In all tests chicks were fed in groups of eight. In the 30-minute assay, blood samples were taken 30 minutes after termination of the feeding period. For the 6-hour assays the feed was offered on either an ad libitum basis or by a "controlled" feeding procedure. This latter procedure provided a small quantity of feed every 30 minutes for 12 consecutive feedings (3). Blood samples were obtained 30 minutes after the last feeding. For these studies the valine and lysine requirement of the chick

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[2] Plasma from each of 8 chicks/treatment were pooled and after centrifuging, a 10-ml aliquot was taken for analysis. After precipitating the protein with picric acid a 2-ml sample was placed on the columns of a Beckman-Spinco automatic amino acid analyzer for analysis.

[3] Complete crystalline amino acid mixture B, as a per cent of the total diet: L-arginine·HCl, 1.21; L-histidine·HCl·H_2O, 0.40; L-lysine·HCl, 1.19; L-tyrosine, 0.45; L-tryptophan, 0.15; L-phenylalanine, 0.50; DL-methionine, 0.35; L-cystine, 0.35; L-threonine, 0.65; L-leucine, 1.20; L-isoleucine, 0.80; L-valine, 0.82; glycine, 1.20; L-proline, 0.20; and L-glutamic acid, 10.0.

were considered to be 0.82% and 0.83%, respectively (1).

RESULTS

In experiments 1 and 2 graded levels of valine and lysine, respectively, were fed on an ad libitum basis over a period of 6 hours. Inspection of the data in table 1 shows that valine did not accumulate in the plasma when the diets were severely deficient in this amino acid, whereas a dietary excess resulted in rapid accumulation of valine in the plasma.

The data for the 6-hour lysine assay are recorded in table 2. In this instance lysine accumulated in the plasma even though the dietary concentration of this amino acid on the three lowest levels was less than the amount needed to maximize growth (1). The plasma lysine pattern and the plasma lysine levels deviate from that

TABLE 1

Effect of ad libitum feeding of diets containing graded levels of valine on plasma amino acids in a 6-hour assay (exp. 1)

Amino acid	Dietary valine, %							
	0.37	0.47	0.57	0.67	0.77	0.92	1.07	1.37
	μg of amino acid/ml plasma							
Threonine	147.1	101.0	157.8	113.8	115.0	126.2	124.3	126.8
Proline	24.9	25.2	20.5	21.7	18.5	19.4	20.8	20.8
Glutamic acid	35.0	57.3	59.2	71.4	66.2	49.4	68.6	60.5
Glycine	73.0	88.0	75.2	101.9	63.4	70.8	77.5	71.6
Cystine	23.7	25.5	23.8	22.5	20.0	23.4	23.2	30.6
Methionine	16.4	20.3	17.9	19.4	14.6	16.0	17.0	16.3
Isoleucine	20.5	22.0	19.3	19.1	11.4	17.6	15.7	18.3
Leucine	28.1	30.5	25.7	25.4	15.5	22.7	20.2	22.0
Tyrosine	20.3	—	11.2	11.9	7.2	16.9	8.4	8.2
Phenylalanine	16.6	—	10.1	10.3	6.3	15.4	7.8	8.5
Lysine	53.5	49.6	42.2	50.3	38.9	52.8	37.4	46.1
Histidine	11.3	11.4	6.7	8.1	6.9	7.5	6.8	7.9
Valine	7.2	7.2	7.9	15.3	18.5	42.3	58.4	99.4
Feed consumed, g/8 chicks	50	64	69	68	70	71	69	74

TABLE 2

Effect of ad libitum feeding of diets containing graded levels of lysine on plasma amino acids in a 6-hour assay (exp. 2)

Amino acid	Dietary lysine, %					
	0.60	0.70	0.80	0.90	1.00	1.10
	μg of amino acid/ml plasma					
Threonine	147.3	133.0	133.6	151.9	121.5	190.4
Proline	—	20.6	21.7	21.0	32.5	23.2
Glutamic acid	51.8	52.2	59.4	44.0	55.2	63.1
Glycine	72.8	94.1	92.9	105.0	81.2	86.1
Valine	30.9	42.1	36.9	29.1	22.9	33.9
Cystine	21.8	20.0	21.8	20.5	18.0	15.9
Methionine	16.9	21.5	19.6	19.0	15.3	16.1
Isoleucine	26.9	33.5	31.3	21.3	22.2	26.7
Leucine	—	34.9	26.9	19.4	17.4	24.6
Tyrosine	17.8	17.5	15.0	11.0	9.0	13.4
Phenylalanine	13.5	14.0	12.0	9.9	7.6	12.0
Histidine	12.6	18.7	15.7	15.6	10.7	15.8
Arginine	70.9	71.9	—	46.5	37.1	56.5
Lysine	23.5	44.9	52.5	58.4	43.9	97.1
Feed consumed, g/8 chicks	76	85	87	83	83	81

observed in long-term (7-day) assays (1) and the 6-hour valine assay reported here.

In both experiments 1 and 2, the concentration of all other amino acids remained essentially constant even though valine and lysine were varied from a deficient to an excess level in the diet.

Marked differences in the voluntary consumption of feed were noted when chicks were fed the experimental diets on an ad libitum basis over a 6-hour period. In experiments 3 and 4 a "controlled" feeding procedure was used in an effort to equalize feed intake.

Examination of the data in tables 3 and 4 shows that the trend of the plasma curves for valine and lysine was essentially the same as that noted in the prior trials. Lysine accumulated in the plasma in a linear fashion from the most deficient to the greatest dietary lysine level fed, whereas valine did not start to accumulate until the dietary concentration exceeded the requirement level. Although differences in feed intake were minor in the lysine assay, chicks fed the 0.4% valine diet refused to consume all feed offered. Lower plasma concentrations of valine and lysine were noted with the "controlled" feeding procedure than with ad libitum feeding.

TABLE 3

Effect of "controlled" feeding of diets containing graded levels of valine on plasma valine concentration in a 6-hour assay (exp. 3)

Dietary valine	Plasma valine	Feed consumed
%	μg/ml	g/8 chicks
0.40	6.3	50
0.60	7.7	65
0.90	28.7	69
1.10	49.6	69

TABLE 4

Effect of "controlled" feeding of diets containing graded levels of lysine on plasma lysine concentration in a 6-hour assay (exp. 4)

Dietary lysine	Plasma lysine	Feed consumed
%	μg/ml	g/8 chicks
0.60	9.3	66
0.70	13.3	69
0.80	18.5	68
0.90	36.1	70
1.00	44.3	70
1.20	60.7	69

If the response noted in experiments 2 and 4 is peculiar to lysine, rather than a function of feeding time, shortening the length of the feeding period to something less than 6 hours should not alter the plasma valine curve. To explore this hypothesis a direct comparison between valine and lysine was made in experiment 5 where groups of chicks were restricted to a feeding period of 30 minutes. The treatments included diets containing 2 levels of valine or lysine below the requirement level and 2 levels of each amino acid above the requirement (1). These data are recorded in table 5. As a result of having shortened the feeding phase of the assay to 30 minutes valine accumulated in the plasma in a linear manner. The reason for the erratic response noted for lysine is not readily apparent; however, bleeding time in relation to the chicks' feed consumption pattern may be involved at this point also.

DISCUSSION

The data indicate that the length of the experimental feeding period will alter the shape of the plasma amino acid curve when diets containing graded levels of single amino acids are fed. This alteration in the plasma amino acid response curve was first noted for lysine in the 6-hour assay but it was also apparent for valine in the 30-minute assay. These data suggest that the metabolic system responsible for protein synthesis had not been sufficiently activated in the 6-hour lysine assay and 30-minute valine and lysine assays to utilize all of the first-limiting amino acid that is available in the blood. It might be ques-

TABLE 5

Effect of ad libitum feeding of diets containing graded levels of valine or lysine on the plasma concentration of valine and lysine, respectively, in a 30-minute assay (exp. 5).

Treatment	Plasma amino acid	Feed consumed
%	μg/ml	g/8 chicks
0.40 valine	10.2	22
0.60 valine	25.2	19
0.90 valine	43.8	16
1.10 valine	56.3	18
0.50 lysine	50.0	15
0.70 lysine	44.8	17
1.00 lysine	84.2	15
1.20 lysine	84.0	20

tioned why the system is functioning at 6 hours for valine but not for lysine. Differences in absorption rate deserve consideration as a possible explanation. It is also possible that there is a greater requirement for valine in re-establishing a condition of anabolism in the chick after having been fasted for 4 hours. Lysine might not be required to any degree until true tissue accumulation (growth) occurs. Regardless of the mechanism involved, it appears to be clear that the duration of the feeding phase of plasma amino acid studies must be considered in relation to the objective of the assay. For example, the 6-hour assay would not be satisfactory for determining the lysine requirement of the chick, whereas the longer assays reported previously (1) can be used effectively for this purpose. In the 6-hour assay for lysine, but not valine, and the 30-minute assay for both lysine and valine, the plasma pattern appears to reflect the dietary concentration of these amino acids. McLaughlan (5) has also suggested that length of feeding period may be a factor to consider in calculating plasma amino acid ratios (6).

While there was no indication that method of feeding (ad libitum vs. "controlled") had any pronounced effect on the results obtained, it can be demonstrated that chicks will react to severe amino acid deficiencies (imbalances) in a very short time by a reduction in feed intake. In experiment 1 (table 1) plasma valine remained constant at the three lowest levels of valine fed (all deficient), whereas the voluntary consumption of feed (g/8 chicks) was 50, 64 and 69, respectively. In experiment 6 an attempt was made to pinpoint the onset of anorexia as noted in experiments 1 and 3 by recording feed consumption every 30 minutes during the 6-hour feeding period. Examination of the data in table 6 shows that during the first 4 feedings (2 hours), feed intake of chicks fed the diet severely deficient in valine (0.4%), equaled that of chicks fed diets containing greater concentrations of valine. The decrease in voluntary consumption occurred after this time. The fact that a 4-hour fast preceded the offering of the experimental diets may have contributed to the pattern of feed intake noted. Kumta and Harper (4) have reported that rats reduce their voluntary intake of imbalanced diets after a comparable period of time.

TABLE 6

Voluntary consumption of valine-deficient and valine-adequate diets over a 6-hour period following a 4-hour fast (exp. 6)

Time	Diet consumed/8 chicks			
	Dietary valine, %			
	0.4	0.6	0.9	1.1
hours	*g*	*g*	*g*	*g*
0.5	15	14	17	15
1.0	6	5	5	5
1.5	4	5	5	7
2.0	5	5	4	6
2.5	3	5	7	5
3.0	1	5	2	6
3.5	4	6	6	6
4.0	3	5	6	4
4.5	0	5	5	6
5.0	4	4	5	7
5.5	1	5	5	4
6.0	5	3	5	5
Total	51	67	72	76

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Effect of Fasting and of Feeding a Nonprotein Diet on Plasma Amino Acid Levels in the Chick

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ABSTRACT Feeding a nonprotein-containing diet lowered the concentration of essential amino acids in the blood plasma below the level noted when chicks were fasted over a comparable period of time (3, 6, 12 or 24 hrs). With time, several amino acids (methionine, isoleucine, leucine, tyrosine and phenylalanine) but notably lysine, progressively accumulated in blood plasma under fasting conditions. Threonine was unaffected. Plasma lysine also increased with time when chicks were fed a nonprotein diet; plasma threonine decreased and other amino acids were largely unaffected.

The fasting plasma amino acid pattern has been used as a reference (1) in the evaluation of protein quality. However, there are certain factors that should be considered in using this pattern as a point of reference. One such factor is the progressive increase in plasma threonine and lysine (2–5) noted when the fasting period is extended over a period of several hours. Hill and Olsen (4) observed that the plasma amino acid pattern was not appreciably altered as a result of feeding a nonprotein diet over a period of several hours. They expressed the view that this pattern would be more reliable than the fasting pattern as a reference point in studying protein quality.

The primary objective in this study was to gain more specific information with respect to progressive changes in blood plasma amino acid patterns that result when chicks were subjected to (a) a period of fasting, or (b) a feeding regimen involving a nonprotein diet. The handling of chicks during the pretest period as well as the preparation and analysis of blood samples has been described previously (6, 7).

Experimental blood samples were obtained for analysis after chicks had been subjected to the treatments for either 3, 6, 12, or 24 hours. The 6-hour plasma sample in the nonprotein series was inadvertently destroyed. The nonprotein diet was the same as described previously (7) with the mixture of crystalline amino acids omitted.

RESULTS

The results are recorded in table 1. As reported previously (4), lysine continued to increase in the plasma with each extension of the fasting period. Methionine, isoleucine, leucine, tyrosine, phenylalanine, and histidine also accumulated in the plasma in much the same manner as lysine, whereas proline, glutamic acid and arginine were unaffected by the fast. Valine may have accumulated in the late stage of the fast; plasma cystine, unlike methionine, progressively declined with time. Contrary to previous reports (3, 4), plasma threonine did not increase during the fasting period. No reason for this lack of agreement can be advanced at this time but it has been shown by Gray et al. (5) and Hill and Olsen (4) that prefasting treatment will affect the plasma amino acid pattern during the fasting period. The chicks used in the present study had been fed a diet containing a complete amino acid mixture (7) up until the time that the fast was initiated.

In agreement with the results reported by others (4, 8, 9), the feeding of a nonprotein diet lowered the concentration of amino acids in plasma as compared with the plasma amino acid pattern noted when chicks were fasted. With time, tyrosine and lysine tended to accumulate in plasma, whereas threonine decreased at a uniform rate and the remaining amino acids were unaffected.

DISCUSSION

By providing a dietary source of energy, it appears that either the breakdown of

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TABLE 1

Effect of fasting and feeding a nonprotein diet on plasma amino acids

	Treatment						
	3-hr fast	6-hr fast	12-hr fast	24-hr fast	3-hr NPD [1]	12-hr NPD	24-hr NPD
	μg of amino acid/ml plasma						
Threonine	120.5	119.4	119.0	113.8	65.8	41.4	34.0
Proline	29.7	24.6	28.7	28.8	10.6	10.4	10.6
Glutamic acid	40.5	37.4	40.9	44.2	23.1	16.5	17.8
Glycine	41.2	35.5	39.7	43.6	24.4	18.2	27.3
Valine	28.4	27.6	27.1	43.3	13.1	11.5	11.3
Cystine	37.8	25.7	15.9	12.0	20.9	12.5	—
Methionine	8.0	9.8	10.4	15.0	4.0	4.1	7.6
Isoleucine	14.6	19.0	20.6	24.7	6.9	5.4	8.0
Leucine	22.5	27.5	32.7	42.8	9.0	7.6	11.1
Tyrosine	14.1	23.6	39.4	30.9	10.4	22.0	29.9
Phenylalanine	11.7	13.8	15.5	17.6	7.5	8.8	13.6
Lysine	51.5	71.8	118.0	131.1	19.6	41.0	40.3
Histidine	8.9	9.5	14.7	17.9	4.3	7.3	9.0
Arginine	22.0	19.3	20.5	26.1	—	—	—

[1] Nonprotein diet.

tissue was reduced or re-utilization of the amino acids was enhanced. Probably both conditions were operating at the same time and as a result, the plasma concentration of amino acids was reduced considerably below the fasting pattern by feeding the nonprotein diet.

Previously (7) it was reported that amino acids do not accumulate in the blood unless a rather severe dietary amino acid imbalance exists. That fasting also results in the accumulation of plasma amino acids suggests that the endogenous amino acid pattern identified with fasting, is similar to that of an individual fed a severely imbalanced diet.

Since all amino acids are not affected to the same degree by fasting, it would make considerable difference in interpretation of data whether the 24-hour plasma amino acid pattern was used as a point of reference as opposed to the 3-hour pattern in calculating plasma amino acid ratios in accordance with the technique proposed by Longenecker and Hause (1). Clearly, the fasting and nonprotein plasma amino acid patterns have limitations which detract from their usefulness as reference points in plasma amino acid studies. From this it appears that the best plasma reference pattern would be that which results from feeding a diet with a well-balanced amino acid pattern.

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Aspects of Liver Lipid Metabolism in the Biotin-deficient Rat

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ABSTRACT The effect of biotin deficiency on some aspects of lipid metabolism in the liver of female and male rats was investigated. No significant difference was found either in total lipid content or in lipid composition in the liver of biotin-deficient rats of both sexes when compared with biotin-treated controls. Significant changes were observed in the percentage fatty acid composition, namely more 16:1 and 18:2 and less 18:0 fatty acid were found in total liver lipids of the biotin-deficient rats. The incorporation of ^{32}P into liver phospholipids in intact biotin-deficient rats was not altered. The rate of incorporation in vitro of acetate-1-^{14}C into total liver lipids was significantly decreased in biotin deficiency. These data appear to indicate that biotin deficiency in the rat may result in some changes in liver lipid metabolism.

The mode of action of biotin in several enzymatic reactions associated with lipid metabolism has been established by Lane et al. (1, 2). Okey et al. (3) first reported that biotin takes part in the synthesis and storage of fatty acids. It was found later that purified preparations of acetyl CoA carboxylase contained biotin (4), suggesting that the vitamin plays an essential role in the biosynthesis of fatty acids. Furthermore, avidin was shown to inhibit in vitro the conversion of acetyl CoA to palmitate. This inhibition is removed by the introduction of a biotin supplement (4, 5). Gram and Okey (6) have suggested that there may be an inhibition in the synthesis of glycerides and phospholipids in biotin-deficient rats, whereas the data on the participation of biotin in cholesterol metabolism are contradictory (3, 6–8).[1]

In the present paper the effects of biotin deficiency on the composition of liver lipids and on the percentage composition of the fatty acids in the lipids in rat liver are reported. The effects of biotin deficiency on the incorporation in vivo of ^{32}P into liver phospholipids and on the incorporation in vitro of acetate-1-^{14}C into liver lipids are also described.

EXPERIMENTAL

Weanling male and female albino rats of the Wistar strain, weighing 40 to 45 g, were divided into 2 groups, housed in wire-bottom cages and fed ad libitum a biotin-free diet and a biotin-supplemented diet, respectively (table 1). After 60 days some of the animals in each group were killed and the liver was used for the determination of 1) lipid composition; 2) fatty acid composition of total lipids; and 3) biotin and nicotinamide nucleotide content; also for testing lipogenesis in vitro.

Total lipids were measured gravimetrically after extraction by a method described previously (9); cholesterol was analyzed by the method of Sperry and Webb (10); and phospholipids by phosphorus analysis in total lipids (11). Samples of total lipids were saponified with ethanolic KOH. Methyl esters of fatty acids were prepared by refluxing in dry methanolic HCl and determined by gas-liquid chromatography on a Pye apparatus equipped with an argon ionization detector, and with columns with a temperature and a polarity suitable for separation of fatty acids up to 22:6. The area percentage of peak for each component was determined by triangulation.

Biotin was assayed microbiologically with *Lactobacillus arabinosus* 17/5 ATCC 8014 on samples of liver homogenate autoclaved with 5 N H_2SO_4 at 121° for one hour. The microbial growth was determined by titrating the lactic acid formed with 0.1 N NaOH after 72 hours' incubation at 30°. Nicotinamide nucleotide con-

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[1] Jacobsohn, G. M., and R. C. Corley 1957 Formation of cholesterol in liver slices from biotin-deficient rats. Federation Proc., *16:* 200 (abstract).

TABLE 1

Composition of experimental diets

	Biotin-free	Biotin-supplemented
	%	%
Casein, vitamin-free	20	20
Sucrose	59	59
Salt mixture IV [1]	4	4
Peanut oil	5	5
Autoclaved egg white [2]	—	11
Raw dried egg white	11	—
Vitamin mixture [3]	1	1
Biotin	—	0.0001

[1] Hegsted et al. (21).
[2] Autoclaved at 120° for 30 minutes and dried.
[3] The vitamin mixture provided the following/kg of diet: (in milligrams) thiamine·HCl, 2; riboflavin, 2; pyridoxine, 2.5; Ca-pantothenate, 20; niacin, 50; inositol, 100; p-aminobenzoic acid, 250; folic acid, 0.20; choline·HCl, 1000; menadione, 2.1. Two drops of vitamin A, D and E concentrate were fed to each rat once a week. The concentrate contained per gram: vitamin A, 20,000 IU; vitamin D_3, 5,000 IU; and *dl*-α-tocopherol, 100 mg.

tent was determined by the fluorimetric procedure of Dianzani (12) and liver protein by the colorimetric procedure of Lowry et al. (13).

For the experiments on lipogenesis in vitro, liver slices (500 mg) were incubated in 25-ml Erlenmeyer flasks containing 5 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, 5 μmoles of α-ketoglutarate and 0.5 μmoles of sodium acetate-1-^{14}C [2] (specific activity 10.5 mCi/mmole).

The flasks were shaken at 37° for one hour and the reaction was stopped by placing the flasks on ice. After removal of the medium, the slices were rinsed 3 times in ice-cold buffer, and homogenized in 20 vol of 2:1 chloroform-methanol. The extract was evaporated to dryness and the lipid residue was dissolved in light petroleum (40–60 bp) and then in chloroform. Aliquots of this solution were taken for radioactivity measurement in a windowless gas-flow counter.

The remaining animals of each group were injected intraperitcneally with 0.5 mCi of $NaH^{32}PO_4$.[3] The rats were killed after 10 hours and the livers were used for the measurement of the incorporation of ^{32}P into phospholipids as described previously (14). The radioactivity was determined in a well-type scintillation counter with a plastic crystal. The specific radioactivity of each sample was corrected for differences in dosage and body weight. As used in the present study, specific radioactivity is defined as 100 × count/min/mg of phospholipids divided by the counts per minute administered per gram of body weight. To relate the fraction more directly to its precursor, the radioactivity of phospholipid fraction was reported as relative specific radioactivity. The relative specific radioactivity of a fraction is defined as 100 × the specific radioactivity of that fraction divided by the specific radioactivity of the acid-soluble phosphorus. The total relative radioactivity is defined as the amount of phospholipids per gram of defatted liver multiplied by its relative specific radioactivity.

All data were analyzed by statistical procedures.

RESULTS

It appears from table 2 that there is no significant difference either in total lipid content or in lipid composition of the liver of biotin-deficient rats of both sexes as compared with biotin-treated animals.

[2] Obtained from the Radiochemical Centre, Amersham, Bucks, England.
[3] See footnote 2.

TABLE 2

Effect of biotin deficiency on the composition of lipids in rat liver

Group [1]	Experimental treatment	Body wt	Liver lipids			
			Cholesterol	Phospholipids	Neutral fat	Total lipids
		g	*mg/g wet tissue*			
1	Biotin-treated, male	247 ± 15 [2]	2.41 ± 0.17	22.53 ± 0.66	27.44 ± 1.33	52.38 ± 1.28
2	Biotin-treated, female	170 ± 11	2.55 ± 0.22	29.52 ± 1.46	24.58 ± 2.82	56.59 ± 2.73
3	Biotin-deficient, male	165 ± 23	3.04 ± 0.23	26.35 ± 0.95	27.58 ± 0.11	56.97 ± 1.14
4	Biotin-deficient, female	127 ± 19	2.53 ± 0.30	24.77 ± 1.57	29.32 ± 1.81	56.64 ± 2.11

[1] Six rats/group.
[2] Averages ± SE of mean.

TABLE 3

Effect of biotin deficiency on the fatty acid composition of total liver lipids

Fatty acids	% fatty acid in total liver lipids	
	Biotin-treated rat [1]	Biotin-deficient rat
14:0	0.20 ± 0.03 [2]	0.53 ± 0.66
15:0	0.13 ± 0.01	0.66 ± 0.04
16:0	22.05 ± 2.17	22.30 ± 2.47
16:1	2.48 ± 0.30	5.00 ± 0.41
17:0	0.30 ± 0.02	1.47 ± 0.22
18:0 iso	0.23 ± 0.02	0.89 ± 0.10
18:0	25.80 ± 2.35	18.64 ± 2.20
18:1	33.58 ± 2.97	32.09 ± 3.01
18:2	5.30 ± 0.61	8.03 ± 0.70
20:2	0.97 ± 0.11	1.91 ± 0.21
20:4	4.64 ± 0.41	4.76 ± 0.41
20:5	0.36 ± 0.04	0.30 ± 0.02
22:5	0.64 ± 0.08	1.00 ± 0.09
22:6	3.30 ± 0.21	2.40 ± 0.19
Ratios:		
20:4/18:2	0.88	0.59
16-carbon/18-carbon	0.38	0.46
Saturated/unsaturated	1.03	0.85

[1] Six female rats/group.
[2] Average ± SE of mean.

Table 3 shows a comparison of the percentage fatty acid compositions in the total liver lipids of the normal and of the biotin-deficient rat. Biotin deficiency resulted in significant increases in 16:1 ($P < 0.001$) and 18:2 ($P < 0.01$). A significant decrease in 18:0 ($P < 0.01$) was also shown. The ratio of 20:4 to 18:2 is decreased in total liver lipids of biotin-deficient rats. Also, the ratio of saturated to unsaturated fatty acids was slightly but significantly decreased. The ratio of 16-carbon to 18-carbon fatty acids was increased in biotin-deficient animals.

The level of total biotin (table 4) was decreased in biotin-deficient male rats ($P < 0.001$) and decreased to a greater extent in female rats ($P < 0.001$). No significant differences were noted in the content of liver nicotinamide nucleotides between biotin-deficient and biotin-treated animals. Furthermore, no significant difference in liver protein content was observed between the groups.

The effect of biotin deficiency on lipid synthesis in vitro is shown in table 5. In-

TABLE 4

Effect of biotin deficiency on the content of total liver biotin and nicotinamide nucleotides

Group [1]	Experimental treatment	Total liver biotin	Liver nicotinamide nucleotides
		μg/g fresh tissue	*μg/g fresh tissue*
1	Biotin-treated, male	1.83 ± 0.08 [2]	1,280 ± 80
2	Biotin-treated, female	2.94 ± 0.11	1,640 ± 260
3	Biotin-deficient, male	0.34 ± 0.06	1,230 ± 60
4	Biotin-deficient, female	0.47 ± 0.17	1,370 ± 70

[1] Six rats/group.
[2] Averages ± SE of mean.

TABLE 5

Effect of biotin deficiency on the incorporation of acetate-1-^{14}C in vitro in the liver lipids of rat

Group [1]	Experimental treatment	Radioactivity [2]	
		cpm/g wet tissue	*cpm/mg liver protein*
1	Biotin-treated, male	942 ± 198 [3]	4.66 ± 0.09
2	Biotin-treated, female	2,023 ± 356	9.87 ± 0.19
3	Biotin-deficient, male	347 ± 28	1.75 ± 0.02
4	Biotin-deficient, female	428 ± 37	2.07 ± 0.04

[1] Six rats/group.
[2] As used in this study, radioactivity is defined as count/min of acetate-1-^{14}C incorporated into liver lipids, either/g of wet tissue or/mg of liver protein.
[3] Averages ± SE of mean.

TABLE 6

Effect of biotin deficiency on the incorporation of ^{32}P in vivo into liver phospholipids of rats

Group [1]	Experimental treatment	Relative specific radioactivity [2]	Total relative radioactivity [3]
1	Biotin-treated, male	1.89 ± 0.23 [4]	42.6 ± 4.3
2	Biotin-treated, female	1.98 ± 0.27	58.4 ± 6.2
3	Biotin-deficient, male	1.75 ± 0.19	46.1 ± 3.9
4	Biotin-deficient, female	1.77 ± 0.22	43.8 ± 3.7

[1] Six rats/group.
[2] The relative specific radioactivity of a fraction is defined as 100 × the specific radioactivity of that fraction divided by the specific radioactivity of the acid-soluble phosphorus.
[3] The total relative radioactivity is defined as the amount of phospholipids per gram of defatted liver multiplied by its relative specific radioactivity.
[4] Averages ± SE of mean.

corporation of acetate-1-^{14}C into total lipids in vitro was significantly inhibited in biotin-deficient female rats ($P < 0.001$) and to a lesser extent in male rats ($P < 0.02$). In contrast the rate of incorporation of ^{32}P into liver phosphospholipids in vivo did not appear to be significantly altered by biotin deficiency (table 6).

DISCUSSION

Data from these experiments suggest that biotin deficiency in the rat does not influence either the total fat content or the lipid composition of liver. Also, the incorporation of radioactive phosphate into liver phospholipids of intact animals is not significantly affected by biotin deficiency. However, ^{32}P incorporation into phospholipids could conceivably be independent of the synthesis of the fatty acid portion. Further, the possibility cannot be excluded that the time-course of the ^{32}P incorporation into phospholipids might have showed a significant difference. However, it has been shown previously that in intact biotin-deficient chicks, no significant change was observed either in liver fatty acid content or in incorporation of acetate-1-^{14}C into liver lipids when compared with biotin-treated animals (15). In contrast, biotin deficiency markedly decreased liver lipogenesis in vitro and resulted in significant changes in the percentage fatty acid composition in the total liver lipids. The observed decrease of the ratio of the saturated-to-unsaturated fatty acids in the total liver lipids suggests that in biotin deficiency there may be an alteration in the metabolic processes of unsaturated fatty acids. The decrease of the ratio of arachidonate (20:4) to linoleate (18:2), which indicates a decreased transformation of linoleate to arachidonate (16), is consistent with this hypothesis. Also, the increase of linoleate (18:2) could be attributed to decreased conversion to the polyunsaturated higher fatty acids, since it has been demonstrated that linoleic acid cannot be synthesized by the rat (17).

Moreover, in biotin-deficient rats a marked decrease in stearate (18:0) and an increase in palmitoleate (16:1) were observed and the ratio of 16-carbon to 18-carbon fatty acids was increased. These data are in agreement with the observations that yeast reacts to biotin deficiency by synthesizing less 18-carbon and more 16-carbon fatty acids (18) and that also in biotin-deficient chicks a significant increase of palmitoleate (16:1) and a significant decrease of stearate (18:0) in the carcass lipids were observed, whereas oleate (18:1) was unchanged (19). In this respect, it has been suggested that oleate (18:1) is synthesized by an independent pathway which is not affected by biotin deficiency (20).

Modi and Mistry (20) have also shown that in biotin-deficient rats the percentage fatty acid composition of the mitochondrial liver lipid showed a significant decrease in palmitate (16:0) and stearate (18:0).

In conclusion, biotin deficiency in the rat may result in significant changes of liver lipid metabolism. However, while the decrease of liver lipogenesis could be explained by the role of the biotin-enzyme

acetyl CoA carboxylase in the fatty acid synthesizing system (4, 5), the mechanisms responsible for the observed changes in the fatty acid composition of liver lipids in biotin-deficient animals remain to be demonstrated.

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Urinary Amino Acids of Rats Receiving High Sugar Diets [1]

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ABSTRACT The urinary amino acids of weaned rats fed various high pentose and hexose diets were studied. D-Xylose, D-ribose, D-lyxose, D-arabinose, L-arabinose, and D-xylitol at diet levels of 20 to 25% reduced taurine excretion 80 to 90%, whereas other urinary amino acids were only moderately decreased. A pronounced amino aciduria resulted from feeding D-lyxose. With hexose diets, D-galactose and 3-0-methyl-D-glucose were found to have no specific effect on taurine, but D-6-deoxygalactose, inositol and ascorbic acid lowered urinary taurine. Repeated and prolonged intraperitoneal injections of lyxose and xylose did not cause a decrease in taurine output, suggesting an intestinal locus for the action of these sugars. Gut sterilization with neomycin tended to reverse the fall of urinary taurine with xylose diets. Reduced protein intake, diarrhea, and intestinal bloat which accompanied the pentose diets did not appear to be the factors responsible for the decreased excretion of taurine.

Disturbances in sulfur amino acid levels have been frequently reported to accompany experimental cataractogenesis. Studies have focused in particular on glutathione since the concentration of this substance in the lens may exceed that in any other tissue (1). Cataracts developing when high galactose and xylose diets are used are preceded by drastic reductions in lenticular glutathione (2–4). Diabetic and senile cataracts are likewise associated with lowered glutathione or cysteine levels in the lens (5, 6). The severity of radiation-induced cataract appears proportional to the loss of glutathione (7). The aromatic hydrocarbon, naphthalene, which is partly detoxified by conjugation with cysteine, may induce cataracts by a transient depletion of sulfur amino acids (8).

The possibility that more widespread changes in sulfur metabolism occur during cataract formation than those manifested by the lens appears not to have been thoroughly investigated. As an approach to this problem, urinary amino acids were studied in rats fed cataractogenic sugar diets. Various diets containing related sugars and sugar-like substances were also tested. Since taurine represents both the principal sulfur amino and acidic amino compound excreted by the rat (9), it was believed that fluctuations in the level of this compound would provide evidence of any generalized influence on extra-lenticular sulfur amino acid metabolism.

METHODS AND MATERIALS

Female rats of the Holtzman strain were fed the diets four to six days after weaning. This strain is particularly vulnerable to sugar cataracts (10). Diets were prepared by thoroughly mixing the appropriate sugars with powdered laboratory ration.[2] Urine was collected for 24 hours in metabolism cages; a few milliliters of alcohol were added to the collection cylinders as a preservative. Urine for each group of animals was pooled. The animals had access to food during the period of urine collection. The pooled urine was filtered, adjusted to pH 2.0, and aliquots were chromatographed on columns of sulfonated Polystyrene resin with an automatic Spinco Amino Acid Analyzer Model 120B. Since the sulfur amino acids and their nitrogenous metabolites separate with the acidic and neutral amino acids, chromatography was not carried out routinely for the basic amino acids.

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

A sugar supplement was generally added to the food to make the final concentration 20 or 25% since with greater amounts the animals became debilitated or failed to grow. The 35% diets of D-galactose and D-xylose are commonly used for producing cataracts, which usually develop in two or three weeks. Feeding experiments were not ordinarily continued this long since the changes in urinary amino acids were found to occur considerably earlier, and in many cases the sugars were too toxic for prolonged feeding.

Taurine urinary excretion generally ranged from 15 to 25 μmoles/day for different animals. Occasional rats excreting 10 μmoles or less/day were discarded since diets which decreased taurine output sometimes reduced excretion to levels which could not be measured. Although individual variation between animals was not large, most experiments were conducted so that the same group of rats provided excretion data for both normal and sugar supplemented diets. Variation in taurine excretion for the same rat remained small, as determined by analyses both before feeding the sugar diets and two to three days after normal diets were restored to the animals. This variation remained below 10% in 2 groups of 2 animals and below 20% in two other pairs. This retesting was within a 12-day interval.

The data presented were obtained from recently weaned rats. Most of the experiments were repeated with rats weighing 100 g or more with similar results, and hence only the former studies are tabulated.

RESULTS

Table 1 shows the major acidic and neutral amino acids present in a normal urine sample and the same amino acids in urine from animals fed galactose and xylose. Minor components which could not be calculated with sufficient accuracy to note significant changes in daily excretion are not listed. Asparagine and glutamine are grouped together since they are not resolved by the resin.

The outstanding change observed was the marked decrease in taurine excretion with the xylose diets. The other cataractogenic sugar, D-galactose, did not significantly depress urinary taurine. The 5-day test period with galactose was selected since this time-interval of feeding the diet depresses lens glutathione to about 10% of control values (3).

The decrease in urinary taurine with xylose feeding was not due to any experimental or analytical artifact. The taurine peak is one of the first to emerge from the column. The possibility that the urinary sugars were washed from the column simultaneously and somehow suppressed the ninhydrin color reaction with taurine was investigated and eliminated. Adding varying amounts of xylose to control urine did not influence the taurine peaks. Likewise, known amounts of taurine added to xylose urine were recovered after chromatography, the peaks showing no suppression or displacement relative to other amino acids. This indicates the absence of any ninhydrin-taurine color reaction inhibitor in the experimental urine. Urine from rats fed high ascorbic acid diets contained diminished amino acids due to the formation of artifacts in the heating coil of the analyzer.

Some experiments were designed to investigate the possible modes of action of xylose on taurine excretion. Rats fed xylose diets suffer a curtailed caloric intake since this sugar cannot be metabolized. The substitution of xylose for a portion of the diet leads to a reduced consumption of amino acids because the rats make no effort to increase actual food intake to counterbalance the lowered protein and caloric value of their diet (11). In addition, high pentose diets produce diarrhea and distended abdomens owing probably to intestinal fermentation (10, 11). This factor may interfere with the intestinal absorption of nutrients and in conjunction with the preceding factor make pentose-fed rats severely undernourished relative to control animals. Growth, as manifested by weight gain, is much impaired with these sugar diets (10, 11).

The above consideration made it appear plausible that the lowered urinary taurine resulting from xylose diets is a consequence of reduced nutrition. Supporting this idea is the observation that other urinary amino acids are also reduced with

TABLE 1

Urinary amino acids of rats fed cataractogenic sugar diets

Diet	No. of rats	Days fed diet	Taurine	Aspartic acid	Threonine	Serine	Asparagine + glutamine	Glutamic acid	Glycine	Alanine
			μmoles amino acid/24 hr/rat							
Normal [1]	2	16	21.3	0.52	2.93	1.79	4.77	0.64	5.89	2.04
D-Galactose (35%)	3	5	16.2	0.52	2.02	1.76	4.07	1.06	5.13	2.41
D-Xylose (35%)	4	8	1.4	0.31	0.66	0.59	1.69	0.43	1.90	1.15
D-Xylose (18%)	3	8	1.6	0.44	1.53	1.10	2.92	0.65	3.70	2.02
D-Xylose (9%)	3	6	4.3	0.40	0.83	0.79	2.00	0.95	1.91	1.77
Normal, xylose-injected (0.5 g/day)	4	8	20.0	0.57	1.44	1.12	3.51	0.77	2.48	2.48
Normal, xylose-injected (1.0 g/day)	3	5	18.5	0.96	1.20	0.68	1.84	2.26	3.92	3.02
D-Xylose (25%) + 0.5% L-methionine	2	11	35.6	0.72	1.79	1.27	2.83	0.83	2.87	2.98
D-Xylose (25%)	3	28	4.3	0.76	3.36	2.57	3.92	1.20	9.86	3.85
D-Xylose (25%) + 3 days normal diet	3	28+3	42.1	0.81	4.82	3.38	5.01	1.81	13.16	5.82
D-Xylose (35%)	2	50	2.3	0.43	2.00	1.22	3.30	0.45	3.58	1.59
D-Xylose (35%) + 7 days normal diet	2	50+7	30.0	1.26	3.70	2.36	7.29	2.13	5.03	4.49
D-Xylose (25%)	2	0	21.8	0.06	1.07	0.82	2.01	0.47	4.69	0.90
D-Xylose (25%)	2	1	7.8	0.62	0.26	0.66	1.66	0.38	3.25	1.08
Semi-starved [2]	2	6	15.0	0.19	0.54	0.38	1.38	0.13	1.55	0.39

[1] Powdered Laboratory Chow (Ralston Purina Company, St. Louis); protein, 23%; fat, 4.5%; fiber, 6%; ash + minerals, 10%.
[2] Half-ration for 4.5 days, fasted 36 hours.

the xylose diets. Van Heyningen (4) has similarly noted a moderate decrease in some plasma amino acids by paper chromatography in rats fed 35% xylose for several days. This undernutrition hypothesis was explored by determining the urinary amino acids of rats maintained with a normal but restricted diet. The semistarved rats received only one-half the rations consumed by weaned rats fed ad libitum. They were then fasted 12 hours before being placed in metabolism cages, and were not fed during the 24-hour period of urine collection.

Table 1 shows that the semistarved and fasted rats experienced a marked reduction in urinary amino acid levels, of a magnitude at least equal to that for the decrease obtained with xylose feeding, whereas the taurine excretion was depressed only about 30% compared with the 90% observed with the 35% sugar diet.

After semistarvation and fasting for this 6-day interval, the rats were 10% below their 56-g starting weights. Xylose and other sugars fed to rats produced weight gains ranging from 0.9 g to 4 g/day, indicating that the sugar-fed animals were in a better nutritional state than the semistarved controls and that the taurine decline is not directly the result of restricted caloric or protein intake.

Further demonstration that xylose feeding does not cut taurine excretion by simple reduction of protein intake or as a

consequence of diarrhea is obtained by feeding 18% and 9% xylose diets. These mixtures provide more protein for the animal and at the same time greatly diminish the severity of the diarrhea and intestinal bloat. Table 1 shows that taurine excretion was still decreased 80% or more with these diets. Finally, animals given 35% xylose for 4 weeks are reported to become adapted to this regimen (11). Growth and grooming resume and the abnormal softness of the feces is absent (11). In the present study, animals given 25% and 35% xylose for prolonged periods still showed a very marked reduction of urinary taurine (table 1).

Other experiments were carried out in an effort to determine the mechanism by which xylose influenced taurine excretion. Feeding xylose for a single day, during the 24-hour confinement for urine collection, was sufficient to significantly depress taurine excretion. This rapid action precedes any of the long-term effects of xylose feeding such as growth inhibition, bloat, and diarrhea.

The intraperitoneal injection of 2 ml of 25% xylose solution daily for 8 days and twice daily for 5 days into 2 groups of weaned rats was found to be without appreciable effect on urinary taurine. The latter injection schedule gave the animals a gram of xylose each day, a dose comparable to the intake with the 9% diet and probably comparable to the intake on the 18% diet if allowance is made for the poor intestinal absorption of xylose (12, 13) and for the fact that much xylose may be lost because of the diarrhea. The failure of injected xylose to influence taurine excretion suggests that the site of action of dietary xylose is in the intestine.

The addition of 0.5% L-methionine to the xylose diet greatly increased the excretion of taurine. Methionine is readily converted into cysteine in the body and the latter compound is the metabolic source of taurine. The methionine supplement apparently neutralizes whatever inhibitory action the xylose exerts on sulfur amino acid metabolism, transport, or excretion. The addition of methionine did not improve the appearance of the rats or prevent diarrhea or bloat. Weight gain averaged 4 g a day over the 11-day period. Five rats maintained with 25% xylose over a 13-day interval also averaged a 4-g increase/day, and hence methionine supplements did not benefit the animals.

To determine whether the decrease of taurine excretion is a specific result of xylose diets, other pentoses were fed at comparable levels and the results are shown in table 2. D-Ribose, D-arabinose, L-arabinose, and the sugar alcohol, D-xylitol, were all effective in reducing urinary taurine. These substances likewise produced intestinal bloat, diarrhea, and growth retardation. D-Lyxose also lowered taurine levels in the urine but enhanced the excretion of other amino acids. With lyxose feeding for even a few days, an amino aciduria developed that markedly increased most urinary amino acid concentrations. In particular, glutamic acid values increased ten to twenty times control values. Lyxose appeared toxic to rats and survival beyond a few days of feeding was difficult to achieve. The intraperitoneal injection of lyxose did not interfere with taurine excretion and did not elevate concentrations of other amino acids in the urine.

The weight gain of animals fed 20% pentose diets varied considerably. With L-arabinose for 5 days, weight increase averaged 2.3 g/day. With D-ribose for 6 days the increase was 1.2 g/day. Lyxose feeding for 4 days resulted in an average increase of 0.9 g a day. Xylose and galactose at 25% diet levels permitted weight gains of 4 g or more each day.

Table 3 shows urinary amino acid values for some rats fed hexose. Results with glucose are not shown since excretion was highly variable, probably because this is a normal nutritional substance to which the rat could make metabolic adaptations and because the diet already contained high concentrations of metabolic sugar. The results with ascorbic acid were also unclear since this substance reacted with amino acids during the heating process in the analyzer with the consequent destruction of some amino substances and the production of artifacts. D-6-Deoxygalactose lowered taurine, unlike the parent compound, and also increased glutamic acid in the urine, somewhat like lyxose. This compound appeared fairly

TABLE 2

Urinary amino acids of rats fed pentose diets

Diet	No. of rats	Days fed diet	Taurine	Aspartic acid	Threonine	Serine	Asparagine + glutamine	Glutamic acid	Glycine	Alanine
						μmoles amino acid/24 hr/rat				
Normal	3	—	21.3	tr	1.61	1.24	2.73	0.66	5.17	1.70
D-Xylose (25%)	3	5	4.7	1.29	0.29	1.41	3.07	0.69	5.87	2.30
Normal	2	—	35.3	0.58	1.75	1.44	3.10	1.09	4.73	3.80
L-Arabinose (20%)	2	5	2.3	0.47	0.68	0.87	1.67	2.04	1.85	2.44
Normal	3	—	28.1	0.53	1.72	2.78	3.31	0.91	3.60	3.40
D-Ribose (20%)	3	6	3.9	0.48	0.60	0.76	1.34	1.52	1.34	2.56
Normal	2	—	23.6	0.30	1.99	2.03	3.92	0.18	2.89	2.17
D-Xylitol (20%)	2	3	6.1	2.21	2.11	2.37	2.13	9.45	5.80	5.70
D-Lyxose (20%)	2	1	0.9	0.59	0.60	0.75	0.75	2.39	2.92	1.34
D-Lyxose (20%)	2	2	2.6	0.79	0.75	1.08	1.12	2.80	2.72	2.88
D-Lyxose (20%)	2	3	2.7	1.73	1.52	1.91	2.34	7.60	4.69	5.00
Normal, lyxose-injected (0.4 g/day)	2	4	12.4	0.49	1.26	0.93	1.94	1.21	3.98	1.91
L-Arabinose (25%)	3	14	1.1	0.23	0.78	0.84	2.13	0.36	1.93	1.26
D-Arabinose (25%)	3	8	2.0	0.61	0.91	1.19	2.53	1.09	3.00	2.66
D-Ribose (25%)	3	9	1.9	0.36	0.74	0.67	1.83	0.58	1.48	1.41
D-Lyxose (25%)	2	7	2.2	2.11	2.28	3.08	2.30	17.50	6.07	6.26

TABLE 3

Urinary amino acids of rats fed hexose diets

Diet	No. of rats	Days fed diet	Taurine	Aspartic acid	Threonine	Serine	Asparagine + glutamine	Glutamic acid	Glycine	Alanine
						μmoles amino acid/24 hr/rat				
Normal	2	—	16.7	0.49	3.65	2.24	4.21	0.78	12.62	4.48
D-Galactose (25%)	2	11	37.5	0.70	2.11	2.24	3.63	1.15	14.00	3.96
Normal	2	—	17.6	tr	1.55	1.51	4.11	tr	2.63	1.72
3-*O*-Methyl-D-glucose (25%)	2	3	10.5	0.11	1.85	1.22	2.67	0.41	3.07	1.35
D-6-Deoxy-galactose (25%)	2	7	1.0	1.03	1.26	1.57	1.60	5.81	4.39	2.75
Ascorbic acid (15%)	3	7	2.66	0.23	0.44	0.45	1.28	0.23	0.99	0.63
i-Inositol (25%)	2	13	0.70	0.65	0.73	0.61	1.50	0.89	3.21	0.92

toxic at the levels consumed in the diet. The 3-*O*-methyl-D-glucose, which is not metabolized by the body but is readily transported across the intestinal wall, had only a moderate effect on taurine excretion, which was approximately in proportion to the amount of protein it replaced in the diet.

It has been reported that sterilizing the intestinal tract with antibiotics can lower urinary taurine levels (14). Since many of the sugar diets produced distended ab-

TABLE 4

Influence of neomycin on urinary amino acids

Diet	No. of rats	Days fed diet	Taurine	Aspartic acid	Threonine	Serine	Asparagine + glutamine	Glutamic acid	Glycine	Alanine
						μmoles amino acid/24 hr/rat				
Normal + 1% neomycin	4	3	23.4	0.29	1.37	1.03	2.00	0.11	1.68	1.33
30% D-Xylose + 1% neomycin	4	3	14.8	0.55	1.26	1.55	1.22	3.20	6.45	8.20
Normal + 1% neomycin	2	3	12.3	0.14	1.94	1.56	3.38	0.30	2.79	1.76
20% D-Lyxose + 1% neomycin	2	3	15.6	7.30	6.00	7.05	3.26	23.70	22.00	17.50

domens suggesting an abnormal degree of intestinal fermentation, the antibiotic neomycin sulfate was added to xylose and lyxose diets to determine whether the changes produced by these sugars in urinary amino acids could be attributed to the action of intestinal microorganisms. Table 4 indicates the results. Taurine excretion was not as strongly inhibited when neomycin was present in the xylose diet. However, the neomycin did not prevent the diarrhea and intestinal bloat. The lyxose-induced amino aciduria was likewise not prevented by the antibiotic.

DISCUSSION

It is evident from the results that a direct correlation does not exist between sugar cataractogenesis and urinary taurine. Of the two major cataractogenic sugars, only xylose exerts an effect on urinary taurine which is already pronounced after a single day of feeding. Galactose, however, does not significantly lower urinary taurine and in some cases may even increase excretion, as shown in table 3.

D-Lyxose diets have been reported to produce lenticular opacities (10) and L-arabinose may act synergistically with other sugars to produce cataracts (15). Both these sugars lower urinary taurine but other pentoses with no known action on the lens also reduce taurine excretion. It thus appears that the decreased output of urinary taurine observed with some sugar diets is an independent property of these diets and is not related to the ability of sugars to cause cataracts.

The mode of action of these pentoses and some hexose-like substances on taurine excretion is not apparent. The effect is not related to decreased caloric or protein intake as taurine levels are depressed even when the sugar concentration in the diet is greatly reduced; nor is taurine excretion severely decreased in semistarved and fasted animals. The intestinal fermentation and diarrhea occurring with the pentose diets does not appear to be a causative factor in decreasing urinary taurine since animals adapted for several weeks to xylose no longer show these symptoms, yet continue to manifest decreased taurine excretion. Likewise, experimental rats fed diets with decreased pentose concentrations do not suffer these intestinal symptoms but still excrete less taurine.

The site of action of the pentoses appears to be the intestine. The evidence for this conclusion is based on the observation that intraperitoneally injected xylose and lyxose do not significantly depress taurine excretion. Passage through the digestive tract appears to be essential if the sugars are to produce their action on the taurine output.

The role of intestinal microorganisms remains unclear. Changes in levels of intestinal flora appear likely in view of the abdominal bloat accompanying the pentose diets. Yet fermentative activity does not appear to contribute to the depressive action of the sugars on taurine excretion since taurine levels remain low even in rats showing no intestinal symptoms, as described above.

An attempt was made to sterilize the guts of the experimental animals by adding 1% neomycin to the sugar diets.

Bloated abdomens and soft feces persisted, yet taurine excretion was no longer strongly depressed. The level of neomycin fed appeared to be adequate since in the control experiments urinary glutamic acid was sharply reduced. This amino acid is not ordinarily present in freshly voided urine and is formed by bacterial degradation of urinary glutamine (16).

A number of in vivo perfusion studies and in vitro everted-sac studies have shown that sugars may inhibit intestinal transport of amino acids including methionine (17–20). The rate of absorption or transport across the intestinal wall did not relate directly to any action on urinary taurine. Galactose and 3-0-methyl-D-glucose are actively transported and did not depress taurine levels, yet 6-deoxy-D-galactose which is also actively transported produced a decrease in taurine excretion.

The amino aciduria produced by lyxose involved other amino acids ordinarily present in the urine in small amounts and not shown in the tables. Valine, the leucines, and the aromatic amino acids increased five to ten times over control values. The action of lyxose was manifest within two or three days. Neomycin appeared to reverse the taurine depression but did not prevent the amino aciduria. Apparently the action of this sugar was confined to the intestine since injection did not result in a pronounced amino aciduria.

Preliminary work on liver and serum amino acids in rats fed lyxose and other sugar diets was inconclusive. Considerable variation was noted in amino acid concentrations since the animals could not be fasted if the immediate effects of the sugar diets were to be explored. Taurine tended to be low in most liver samples from rats fed pentose and hexose.

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Metabolism of Linoleic Acid in Relation to Dietary Monoenoic Fatty Acids in the Rat [1,2]

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ABSTRACT To study the interaction of dietary monoenoic fatty acids with the metabolism of essential fatty acids, weanling rats were fed a fat-free diet supplemented with oleic and linoleic acids in several ratios. The fatty acid composition of liver lipids was determined by gas-liquid chromatography. The conversion of oleic to eicosatrienoic acid was inhibited by dietary linoleic acid at all dietary levels of oleic acid investigated. The metabolism of linoleic acid could be influenced only when extremely high ratios of oleic to linoleic acid were fed. Petroselinic and erucic acids, representing isomers and homologues of oleic acid, were also fed as possible inhibitors of the metabolism of linoleic acid. Petroselinic acid interfered with the metabolism of linoleic and oleic acids, whereas erucic acid had little effect.

Oleic acid is converted to eicosatrienoic acid (20:3ω9)[3] in essential fatty acid (EFA) deficiency in rats (2). This conversion is inhibited when sufficient amounts of either linoleic, linolenic or arachidonic acid are supplied with the diet (3). Linoleic and linolenic acids, however, inhibit each other's conversions to long-chain polyunsaturated fatty acids when fed simultaneously (4, 5). It appears that linolenic acid is converted preferentially over linoleic, and both dietary linolenic and linoleic acids over endogenous oleic acid (6).

There are indications, however, that dietary oleic acid itself can also act as an inhibitor of the conversion of linoleic to arachidonic acid. Oleic acid fed to guinea pigs at a level of about 8% of calories significantly interfered with the utilization of the small amounts of linoleic acid present in dietary coconut oil (7). In rats, inhibition was indicated by the triene-to-tetraene ratio of fatty acids from liver lipids being higher when olive oil (81% 18:1ω9, 4.5% 18:2ω6) was the only dietary fat than when a dietary oil containing 10% linoleic acid was fed (8). Lowry and Tinsley (9) recently reported interactions of oleic and linoleic acids when fats of varying content of these fatty acids were fed to rats. However, dietary levels of 0.5% of calories of linoleic acid combined with dietary oleic acid in amounts 4 to 35 times as high did not show an inhibitory effect of oleic acid upon the transition of linoleic to arachidonic acid when changes in the product-to-precursor ratio 20:4ω6 to 18:2ω6 were considered. Only when scatter diagrams of these product-to-precursor ratios for individual animals were treated mathematically could an inhibitory effect of increasing amounts of dietary oleic acid upon the conversion of linoleic to arachidonic acid be deduced.

Our communication contributes data describing the interaction of dietary oleic acid and other monoenoic acids with the metabolism of linoleic acid in lipids of rat tissues when varying amounts of both fatty acid esters in highly purified form were fed in different ratios. Results have been summarized briefly in a symposium of the American Institute of Nutrition in 1964 (10).

EXPERIMENTAL

Experiment 1. Animals and diets. Weanling male rats, 24 days old, of the Sprague-Dawley strain, were maintained with a diet containing vitamin-free casein (18%), cellulose (4%), sucrose (74%), and the necessary salts and vitamins (3). Ethyl oleate was substituted for the corresponding caloric amounts of sucrose in

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[3] For explanation of shorthand notation, see ref. (1).

the basic diet and ethyl linoleate was given orally by micro-syringe. Groups of 6 animals each were fed either the fat-free diet or supplements of 1.0, 2.0, 3.9 and 7.6% of calories of ethyl oleate in all possible combinations with zero, 0.2, 0.9 and 3.8% of calories of ethyl linoleate as indicated in table 1. The actual content of linoleic acid in the fat-free diet was 18 mg/kg, which is equivalent to 0.004% of total calories. The daily food intake of the animals was recorded, and the percentage of calories of linoleate fed was calculated for each animal. After 84 days the animals were killed by ether anesthesia. Livers, hearts, brains, testes and epididymal fat pads were quickly removed and kept in saline solution at $-20°$ until they were analyzed.

Ethyl oleate and ethyl linoleate were prepared in our laboratory by standard procedures. The purity of the ethyl oleate was 99.8%, and of the ethyl linoleate 97.7%, containing 2.3% oleate.

Analytical methods. Tissues were homogenized and the lipids extracted with chloroform/methanol (2:1). The lipids were transesterified by refluxing with 30 volumes of a 5% solution of HCl in methanol, and the methyl esters were analyzed by GLC using a Barber-Colman Model 10 apparatus equipped with an argon ionization detector. The column was 183 cm × 0.6 cm (6 ft × ¼ in.) o.d., packed with 20% ethylene glycol succinate [4] (EGS) + 2% phosphoric acid coated on Gas Chrom P,[5] 80–100 mesh. The flow rate was 60 ml argon/minute, and the temperatures were 250° at the inlet heater and at the detector cell, and 192° at the column. Fatty acids up through 18:1 were calculated from data recorded on a 10-mv strip chart recorder, and those beyond 18:1 were calculated from data recorded on a 1-mv recorder. This procedure allows simultaneous analysis of major and minor components without requiring attention of the operator during the GLC run.

The individual esters were designated by equivalent chain length (11). Quantification was carried out by triangulation. Liver lipids were analyzed for each animal and fatty acids from 14:0 to 22:6ω3 were calculated. The fatty acid composition is reported in area per cent for averages of 6 animals and is given with the standard deviation. Fatty acid composition of the

[4] Applied Science Laboratories, State College, Pennsylvania.
[5] See footnote 4.

TABLE 1

Content of oleic acid, linoleic acid and their metabolites in liver lipids in response to varying ratios of dietary oleic and linoleic acids

Dietary fatty acids		Liver fatty acids					
18:1 ω 9	18:2 ω 6	18:1 ω 9	20:3 ω 9	18:2 ω 6	20:3 ω 6	20:4 ω 6	22:5 ω 6
% of calories							
0	0.004	35.6 ± 2.1 [1]	5.6 ± 1.2	0.8 ± 0.2	0.3 ± 0.04	2.6 ± 1.0	0.4 ± 0.2
1.0	0.004	38.3 ± 3.2	5.0 ± 1.0	0.5 ± 0.1	0.2 ± 0.1	2.3 ± 0.7	0.3 ± 0.1
2.0	0.004	36.8 ± 2.8	7.5 ± 0.9	1.1 ± 0.4	< 0.1	3.6 ± 0.8	0.6 ± 0.2
3.9	0.004	36.1 ± 3.8	8.5 ± 2.9	1.1 ± 0.2	< 0.1	2.5 ± 1.2	0.6 ± 0.2
7.6	0.004	43.6 ± 1.6	6.1 ± 1.3	1.0 ± 0.4	< 0.1	2.1 ± 0.3	0.4 ± 0.2
1.0	0.2	37.7 ± 3.3	4.2 ± 0.5	1.6 ± 0.4	0.8 ± 0.3	3.9 ± 1.3	0.8 ± 0.3
2.0	0.2	33.9 ± 1.4	5.6 ± 1.3	2.0 ± 0.5	0.9 ± 0.3	5.3 ± 0.7	0.9 ± 0.3
3.9	0.2	38.0 ± 1.4	4.2 ± 0.4	1.6 ± 0.3	< 0.1	3.4 ± 0.3	0.6 ± 0.1
7.6	0.2	39.2 ± 1.5	4.1 ± 1.0	1.3 ± 0.2	< 0.1	2.7 ± 0.4	0.5 ± 0.1
1.0	0.9	33.8 ± 1.8	1.8 ± 0.8	2.6 ± 0.3	1.1 ± 0.2	7.4 ± 1.7	1.9 ± 0.5
2.0	0.9	32.6 ± 3.3	2.1 ± 0.5	2.6 ± 0.5	1.1 ± 0.3	6.9 ± 1.3	1.4 ± 0.4
3.9	0.9	34.9 ± 2.7	2.5 ± 1.0	2.7 ± 0.8	1.0 ± 0.3	7.5 ± 1.4	1.8 ± 0.5
7.6	0.9	38.9 ± 2.7	1.6 ± 0.5	2.2 ± 0.4	0.5 ± 0.2	5.7 ± 0.2	1.2 ± 0.3
1.0	3.8	28.8 ± 3.9	0.6 ± 0.1	7.0 ± 2.8	0.9 ± 0.2	10.2 ± 2.3	3.7 ± 1.0
2.0	3.6	30.4 ± 3.0	0.7 ± 0.3	5.7 ± 1.0	1.0 ± 0.4	9.9 ± 2.9	3.0 ± 1.0
3.9	3.7	30.2 ± 2.7	0.8 ± 0.4	6.3 ± 1.6	1.0 ± 0.4	10.9 ± 2.1	3.7 ± 1.2
7.6	3.6	32.7	0.3	4.2	0.4	8.3	2.3

[1] The figures are averages of 6 values of area per cent from GLC analyses with their standard deviations. Fatty acids not included in this table showed no consistent trend or were not pertinent to the arguments.

other organs was determined after pooling the tissues according to groups.

Experiment 2. This experiment was started after the results of experiment 1 had shown that higher ratios of oleate to linoleate would be required to show more clearly the influence of oleic acid upon metabolism of linoleic acid. The interactions of two other monoenoic fatty acids with the conversions of linoleic acid were also investigated. Oleic acid, petroselinic acid (18:1ω12), an isomer of oleic acid, and erucic acid (22:1ω9), a homologue of oleic acid, were used as possible antagonists of linoleic acid. The influence of dietary palmitoleic acid upon the conversion of linoleic acid has been studied in a less detailed experiment (12).

Animals and diets. The 3 fatty acids were fed to 24-day-old weanling male Sprague-Dawley rats in the form of triglycerides, substituting for the corresponding caloric amounts of sucrose in the diet described in experiment 1. Ethyl linoleate was given by micro-syringe. Groups of 6 animals each were fed a constant level of 0.5% of calories of ethyl linoleate in combination with triolein, tripetroselinin or trierucin at levels of 1.0, 2.0, 3.9, 7.6, 14.5 and 21.6% of calories. In addition one group was fed a fat-free diet and another was fed 0.2% of calories of ethyl linoleate and 1.0% of calories of triolein. After 65 days on the dietary regimen the animals were killed and the livers removed.

Dietary lipids. Triolein was obtained from The Hormel Institute Laboratory for Lipid Preparation. Purity was better than 99%. Petroselinic acid and erucic acid were isolated from carrot seed oil and rapeseed oil, respectively, by low-temperature crystallization of the free acids and fractional distillation of the esters. The purity of various fractions of 18:1ω12 was between 92 and 98%, containing 16:0 and 18:0 as major impurities. Purity of erucic acid was greater than 95%. Linoleate could not be detected in either preparation. The triglycerides of both fatty acids were prepared from the methyl esters and triacetin by transacylation.

Analytcial methods. Methyl esters of liver lipid fatty acids were prepared by transesterification of the extracted lipids with HCl-methanol and were analyzed by GLC using a Beckman GC2A apparatus equipped with hydrogen flame detector. A 183-cm (6 ft) aluminum column of 0.6 cm (¼ in.) o.d. was packed with 20% EGS + 2% phosphoric acid on Gas Chrom P, 80–100 mesh. Conditions of GLC analysis were as in experiment 1. GLC analyses of pure fatty acid mixtures of known concentrations showed that peak areas were proportional to the relative amount of fatty acids in the model mixtures.

RESULTS

Experiment 1. The rats with oleate-linoleate supplements showed approximately equal growth during the 84 days of the experiment. The relative amounts of oleic, linoleic acid and their metabolites in liver lipids are shown in table 1. The content of oleic acid in liver lipids increased with increasing amounts of dietary oleic acid at each level of dietary linoleic acid. The level of 20:3ω9 was increased only in liver lipids of rats that received no linoleic acid in their diet. The amount of 20:3ω9 was influenced to a much greater degree by varying amounts of dietary linoleic acid; in fact, it decreased tenfold between the dietary levels of zero and 3.6% of calories of linoleic acid. Increasing amounts of dietary linoleic acid gave rise to higher levels of linoleic acid in liver lipids. The levels of the metabolites of linoleic acid, 20:3ω6, 20:4ω6 and 22:5ω6 also clearly indicated the increase of dietary linoleic acid. Within groups of equal intake of linoleic acid, the levels of metabolites of linoleic acid changed rather erratically with increasing amounts of dietary oleic acid, although a trend to lower values of these fatty acids with higher intake of oleic acid may be observed. The effect of increasing amounts of dietary oleic acid upon 20:3ω6 was particularly pronounced at low levels of linoleic acid intake. As dietary oleic acid increased, 20:3ω6 could barely be detected in GLC analysis. These observations do not agree with conclusions of a recently published experiment in which a higher amount of 20:3ω6 was reported in rat liver lipids when a diet rich in oleic acid was fed (13).

The analyses of heart and testes lipids revealed the same general pattern of

changes in fatty acid composition. Dietary linoleic acid exerted a much greater influence upon its metabolites and upon 20:3ω9 in heart lipids than did dietary oleic acid. However, the trend toward lower levels of 20:3ω6, 20:4ω6 and 22:5ω6 with increasing amounts of dietary oleic acid persisted.

In brain lipids the influence of increasing amounts of dietary linoleic acid was reflected quite clearly by the decrease of 20:3ω9 and increasing amounts of 20:3ω6, 20:4ω6, 22:4ω6 and 22:5ω6. However, the influence of dietary oleic acid could not be detected at all.

Analyses of epididymal fat pads showed that both oleic and linoleic acids were deposited in increasing amounts as they were increased in the diet. It appears, however, that increasing amounts of dietary oleic fed with the same amount of linoleic acid tended to lower the deposition of linoleic acid.[6]

Experiment 2: Oleic acid. The increase of dietary oleic acid from a maximal 7.6% of calories in experiment 1 to 21.6% of calories, representing a ratio of oleic to linoleic acid of 46:1 in this experiment, did not significantly change the pattern of fatty acids in liver lipids. There was an increased amount of 18:1ω9 deposited (fig. 1). The content of 18:2ω6 and 20:4ω6, however, was not affected appreciably by increasing amounts of dietary oleic acid. The other metabolites of linoleic acid changed rather erratically and a clear trend could not be observed. With the much higher dietary levels of oleic acid in this experiment, however, a significant increase in 20:3ω9 content of liver lipids was observed. The augmented content of oleic acid in liver lipids appeared to be partially compensated for by a decreased content of 16:1.

Petroselinic acid. The dietary fatty acid was deposited in liver lipids, for the final concentration of 18:1 was as high as when triolein was fed. GLC separation of the 2 isomers, 18:1ω9 and 18:1ω12, was not possible under the conditions of our analyses; thus the isomer composition of the 18:1 peak cannot be given. Sand and co-workers (14) recently have shown, however, by analysis of isomers that dietary petroselinic acid is, in fact, deposited in rat liver lipids. After feeding 18:1ω12 at a level of 6% of calories for 3 weeks, 30% of the 18:1 peak in liver lipids consisted of petroselinic acid (14).

The concentration of linoleic acid in liver lipids stayed essentially constant, whereas 20:4ω6 showed a definite decrease

[6] The analytical data for heart, testes, brain and epididymal fat pads are available upon request.

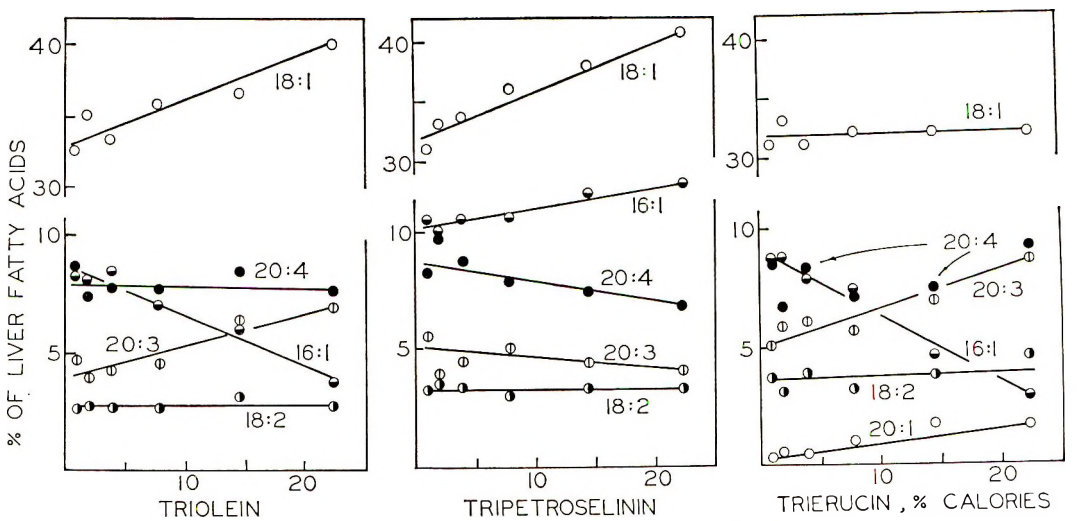

Fig. 1 The content of some fatty acids of rat liver lipids as a function of increasing amounts of dietary triolein, tripetroselinin and trierucin. Fatty acids not included in this figure showed no consistent trend or were not pertinent to the arguments.

with increasing amounts of dietary petroselinic acid. A similar decrease occurred in 20:3ω9. The other metabolites of linoleic acid were not altered significantly. Palmitoleic acid, which decreased when increasing amounts of oleic acid were fed, showed a significant change in the opposite direction with increasing levels of petroselinic acid. According to Sand et al. (14), 16:1ω12 is accumulated in rat liver lipids after partial oxidation of the dietary fatty acid when petroselinic acid is fed.

Erucic acid. This homologue of oleic acid does not appear to become incorporated in liver fatty acids in significant amounts. This has also been shown recently by Carroll (15) who injected radioactively labeled erucic acid into rats. However, a peak identified as 20:1 increased from 0.2 to 1.6% of the total fatty acids when dietary trierucin was increased to 21.6% of calories, probably due to a partial degradation of the dietary acid. The concentrations of oleic, linoleic and arachidonic acids did not change consistently with increasing amounts of dietary 22:1ω9. The concentration of 16:1, however, was markedly lowered from 9.5% to 2.9% of the total fatty acids, whereas 20:3ω9 was increased significantly.

DISCUSSION

The inhibitory effect of dietary linoleic acid upon the metabolism of oleic acid can clearly be deduced from the data of table 1 and the results of experiment 2 in figure 1. Plotting dietary linoleate *versus* average values of 20:3ω9 showed that the conversion of 18:1 to 20:3ω9 is inhibited 50% by approximately 0.6% of calories of linoleic acid in the diet. This agreed with earlier computations in which the inhibition of the metabolism of oleic acid was studied using *endogenous* oleic acid (10). The product-to-precursor ratio for the conversion of 18:1ω9 to 20:3ω9 was calculated in order to judge the inhibition by linoleic acid independently of the changes in the deposition of oleic acid. Table 2 shows that, at all 4 levels of dietary oleic acid, increasing amounts of dietary linoleic acid decreased this ratio significantly.

The effect of dietary oleic acid upon the metabolism of linoleic acid can best be observed at very low levels of dietary linoleic acid. The decrease of 20:3ω6 at 0.004 and 0.2% of calories of dietary linoleic acid and the decrease of 20:4ω6 at the lowest dietary level suggest that dietary oleic acid inhibits the conversion of linoleic to the polyunsaturated fatty acids of the ω6 family. However, the inhibition of the metabolism of linoleic by oleic acid is of a much smaller order of magnitude than the opposite interaction. Levels as high as 20% of calories of oleic acid in the diet could not significantly inhibit the synthesis of 20:4ω6 if the level of dietary linoleic acid was higher than 0.5% of calories.

In figure 2 the triene-to-tetraene ratio (15) was plotted *versus* the ratio of dietary oleic to linoleic acid for heart and liver fatty acids. A definite increase in the ratio of 20:3ω9 to 20:4ω6 could be observed as the ratio of dietary oleic to linoleic acid was increased in those groups which were fed the low levels of linoleic acid. In the group fed fat-free diet only (0.004% of 18:2 calories) the response was somewhat erratic, possibly due to differing reserves of essential fatty acids in the weanling animals. At dietary levels

TABLE 2

Effect of dietary linoleic acid upon the product-to-precursor ratio, 20:3 ω 9/18:1 ω 9, in liver lipids at 4 levels of dietary oleic acid

Dietary 18:1	Dietary 18:2, % of calories			
	0.004	0.2	0.9	3.6
% of calories				
1.0	13.2±3.8 [1]	11.5±1.7	5.3	1.9±0.4
2.0	20.0±4.0	17.0±4.0	6.5±2.0	2.2±1.0
3.9	20.0±3.0	12.0±1.0	7.2±3.0	2.7±1.0
7.6	14.0±3.0	10.5±3.0	4.2±1.5	0.8±0.1

[1] The values for the product-to-precursor ratio were multiplied by 100 and are given with their standard deviations.

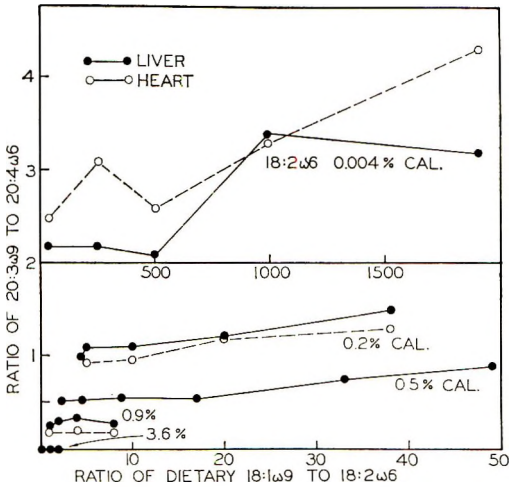

Fig. 2 The relationships between dietary ratio of 18:1ω9 to 18:2ω6 and the triene-to-tetraene ratio of fatty acids of heart and liver lipids at five levels of dietary linoleic acid. Points are averages of 6 values.

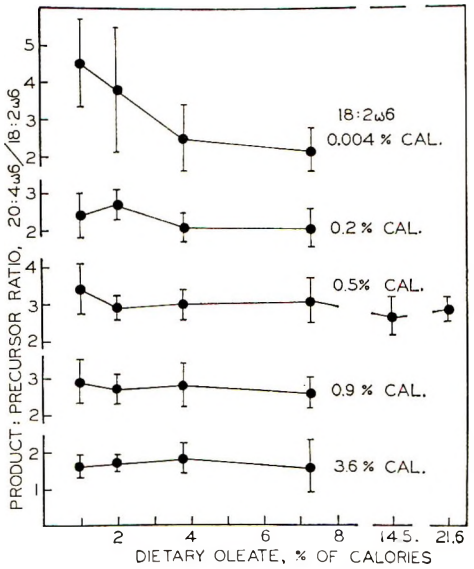

Fig. 3 The relationship of dietary oleic acid and the product-to-precursor ratio of the conversion of linoleic to arachidonic acid in liver lipids. Points are averages of 6 values with standard deviations.

of 0.2 and 0.5% of calories of linoleic acid, the response was consistently demonstrated over a rather wide range of oleic-to-linoleic acid ratios in the diet. The effects were enhanced when ratios of oleic to linoleic acid were above 20 to 1. At 0.9% of calories of linoleic acid and above, the effect of dietary oleic acid was no longer observed.

The inhibitory effect of oleic acid upon the metabolism of linoleic acid was also indicated by the product-to-precursor ratio of 20:4ω6 to 18:2ω6 in liver lipids as plotted in figure 3 for five different levels of dietary linoleic acid *versus* dietary oleic acid. This parameter was significantly depressed by dietary oleic acid at levels of dietary 18:2ω6, equaling 0.004, 0.2 and 0.5% of calories.

In summary, it can be stated, however, that dietary oleic acid fed in proportions as high as 22% of calories of the diet will not significantly affect the conversion of linoleic to arachidonic acid if linoleic acid is fed at or above the minimal requirement of about 1% of calories. Higher intake levels of oleic acid can probably be tolerated if the content of linoleic acid in the diet is increased proportionally.

Petroselinic acid interfered with the conversion of 18:2ω6 to 20:4ω6. The product-to-precursor ratio for this conversion also decreased with increasing amounts of dietary petroselinic acid. The plot of this ratio, in fact, runs parallel to the 20:4ω6/18:2ω6 ratio when triolein is the dietary 18:1. With increasing amounts of dietary petroselinic acid the content of 20:3ω9 in liver lipids decreased. It appears, therefore, that the 18:1ω12 inhibited the conversion of 18:2ω6 to 20:4ω6 as well as the synthesis of 20:3ω9 from endogenous 18:1ω9, without being converted itself to polyunsaturated fatty acids.

Erucic acid was not deposited in liver lipids in appreciable amounts, nor did it appear to influence the conversion of linoleic to arachidonic acid. Changes in 20:4ω6 were rather inconsistent, and the concentration of linoleic acid in liver lipids did not change significantly. There was, however, a significant increase of 20:3ω9 in liver lipids with increasing amounts of dietary erucic acid, which suggests an increased need for PUFA.

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Metabolism of Linoleic Acid in Relation to Dietary Saturated Fatty Acids in the Rat [1,2]

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ABSTRACT To study the interactions of dietary saturated fatty acids with the metabolism of essential fatty acids, triglycerides of saturated fatty acids of chain lengths C_4 to C_{16} were fed in a semipurified diet to groups of weanling rats at 20% of calories for 80 days. All groups were given a daily oral supplement of ethyl linoleate at a level of 0.5% of total calories. The fatty acid composition of liver lipids was determined by gas chromatography. The conversion of linoleic to arachidonic acid was not hindered by the dietary triglycerides of saturated fatty acids. Levels of 20:4ω6 were, in fact, higher in rats fed the triglycerides of saturated fatty acids than in rats fed linoleic acid only. The content of 20:3ω9 was slightly lowered by triglycerides of even-numbered saturated fatty acids of chain lengths up through C_{14}, whereas it is significantly diminished by dietary triglycerides of fatty acids with chain lengths C_5 and C_{11}. Weight gain data revealed that triglycerides of dietary saturated fatty acids enhanced growth if minimal levels of linoleic acid were provided. Odd-numbered fatty acids affected growth adversely.

The conversion of linoleic acid (18:2ω6) [3] to arachidonic acid (20:4ω6) in rat tissues is inhibited by small amounts of dietary linolenic acid (18:3ω3) (2). Oleic acid also interferes with the metabolism of linoleic acid, but the amounts needed for significant inhibition of the synthesis of arachidonic acid are high (3–5). The effects that saturated fatty acids may have upon the metabolism of linoleic acid have been of interest especially with respect to essential fatty acid (EFA) deficiency. A number of reports indicate that saturated fatty acids aggrevated the symptoms of EFA deficiency when fed as the only lipid constituent of the diet (6–8). This could mean that under the conditions of these experiments the small amounts of linoleic acid present in the animal could not be utilized for the synthesis of arachidonic acid or, in other words, the saturated acids in the diet increased the requirement for linoleic acid. Other studies, however, indicate that growth of rats fed saturated fats was not affected (9) nor was even stimulated (10).

The amount of tetraenoic acid present in liver lipids of rats fed hydrogenated coconut oil plus a small dose of linoleic acid was higher than the amount observed in rats fed the same level of linoleic acid only (11). These results indicate that the amounts of dietary linoleic acid determine the effect of saturated fatty acids fed.

In other experiments main emphasis was given to the effects of chain length of dietary saturated fatty acids upon growth of rats. Mixtures rich in stearic, palmitic and myristic acids exerted a growth-depressing effect (12,13), whereas preparations rich in lauric, capric, caprylic and caproic acids stimulated growth of rats (13). Alfin-Slater and co-workers (14) recently have shown that all saturated fatty acids of chain lengths C_4 through C_{18} depress growth when fed singly with an otherwise fat-free diet.

The present investigation was concerned with the influence of individual saturated fatty acids upon the metabolism of dietary linoleic acid when fed simultaneously with different saturated fatty acids.

EXPERIMENTAL

Animals and diets. Male rats, 23 days old, of the Sprague-Dawley strain, were maintained with diets containing vitamin-free casein (18%), cellulose (4%), su-

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[2] This investigation was supported in part by Public Health Service Research Grant no. AM-04524 from the National Institutes of Health; National Dairy Council; and The Hormel Foundation.

[3] For explanation of shorthand notation see ref. (1).

crose (74%), and the necessary salts and vitamins (15). In the basic diet, triglycerides of saturated fatty acids were substituted for the corresponding caloric amounts of sucrose and 20 μliters of ethyl linoleate per day were given orally by micro-syringe. This dose is sufficient to prevent dermal symptoms of EFA deficiency, but does not meet the total requirement for linoleic acid (15). Thus, changes in the metabolism of linoleic acid effected by the dietary saturated fatty acids would produce most significant changes in the triene-to-tetraene ratio of fatty acids of liver lipids.

The fatty acids were supplied in the form of their triglycerides because it is difficult to feed short-chain fatty acids in the form of their very volatile methyl or ethyl esters. Groups of 6 animals each were fed according to the plan shown in table 1. Tristearin was not included in the experiment because, as is well-known, this triglyceride cannot be digested by rats.

The effects of dietary tripalmitolein upon the metabolism of linoleic acid were studied in this experiment because the limited amounts of this fatty acid available did not allow a dose-response study as was conducted previously for the other dietary monoenoic fatty acids (4). "Synthetic butterfat" was prepared from saturated and monoenoic monoacid triglycerides neglecting constituents present in genuine butterfat in amounts of less than 1%.

The rats were maintained with the experimental diet for 80 days and weighed weekly. The animals were killed by ether anesthesia. Livers were quickly removed and kept in saline solution at − 20° until analyzed.

Dietary lipids. Triglycerides were prepared from methyl esters and triacetin by transacylation. Fatty acids were commercial products which were purified by fractional distillation. Palmitoleic acid was isolated from macadamia nut oil by low temperature crystallization of the fatty acids and fractional distillation of methyl esters. Purity of these fatty acids was better than 95%. Myristic, palmitic and palmitoleic acids were better than 99% pure. Ethyl linoleate was prepared from safflower oil and contained 2.3% of oleate as only contaminant.

Analytical procedures. Liver fatty acids were extracted with chloroform-methanol (2:1). The lipids were transesterified with a 5% solution of HCl in methanol. Details of the analytical procedure have been published previously (2, 4, 15).

Methyl esters of rat liver lipids and of dietary lipids were analyzed by gas-liquid chromatography (GLC), using a Beckman GC2A apparatus equipped with hydrogen flame detector. A 183-cm (6-ft) aluminum column, 0.6-cm (¼ in.) o.d., was packed with 20% EGS [4] plus 2% phosphoric acid

[4] Applied Science Laboratories, State College, Pennsylvania.

TABLE 1

Fat supplements to the basic diet

Group	Triglyceride	Constituent fatty acid		Daily supplement of linoleate
			% *of calories*	*μliters*
1	Tributyrin	4:0	20	20
2	Tri-isovalerin	iso–5:0	20	20
3	Trivalerin	5:0	20	20
4	Tricaproin	6:0	20	20
5	Tricaprylin	8:0	20	20
6	Tricaprin	10:0	20	20
7	Triundecanoin	11:0	20	20
8, 9, 10	Trilaurin	12:0	10, 20, 40	20
11	Trimyristin	14:0	20	20
12, 13, 14	Tripalmitin	16:0	10, 20, 40	20
15	Tripalmitolein	16:1	20	20
16	"Synthetic butterfat" [1]		20	20
17	Fat-free		—	20
18	Fat-free		—	—

[1] The "synthetic butterfat" was made up of the monoacid triglycerides of the following acids: 4:0, 3.1%; 6:0, 2.6%; 8:0, 1.2%; 10:0, 3.4%; 12:0, 3.2%; 14:0, 10.0%; 16:0, 26.6%; 18:0, 14.8%; 16:1, 2.0%; 18:1, 33.1%, according to recent analyses of butterfat (16).

on Gas Chrom P,[5] 80–100 mesh. Temperatures were 195° at the column, 250° at the flash heater and at the detector cell. Flow rate was 60 ml helium per minute. Quantification of GLC recordings was carried out by triangulation. GLC analyses of mixtures of known composition showed that peak areas were proportional to the relative amounts of fatty acids in the model mixtures.

The individual esters were designated by equivalent chain length (17). The odd-numbered unsaturated fatty acids, 17:1 and 19:3, which occurred more abundantly in liver lipids of rats fed trivalerin and triundecanoin, were isolated by preparative GLC on a Beckman GC2A apparatus with thermoconductivity detector. Columns and conditions were the same as for analytical runs. The samples collected were identified further by mass spectrometry.

RESULTS AND DISCUSSION

Weight gain. The weight gain of the animals is shown in the upper half of figure 1. All rats fed triglycerides of even-numbered fatty acids gained more weight than rats fed the linoleic acid supplement only. The triglycerides of short-chain fatty acids, tributyrin, tricaproin and tricaprylin, effected greater weight gain than did the triglycerides of medium- and long-chain fatty acids. The triglycerides of odd-numbered fatty acids, trivalerin and triundecanoin, affected the animals adversely. These rats gained less weight than animals maintained with the fat-free diet.

The influence of different caloric amounts of saturated fatty acids is shown for dietary trilaurin and tripalmitin. Whereas feeding of diets containing these 2 triglycerides at 20% of calories supported growth better than linoleic acid

[5] See footnote 4.

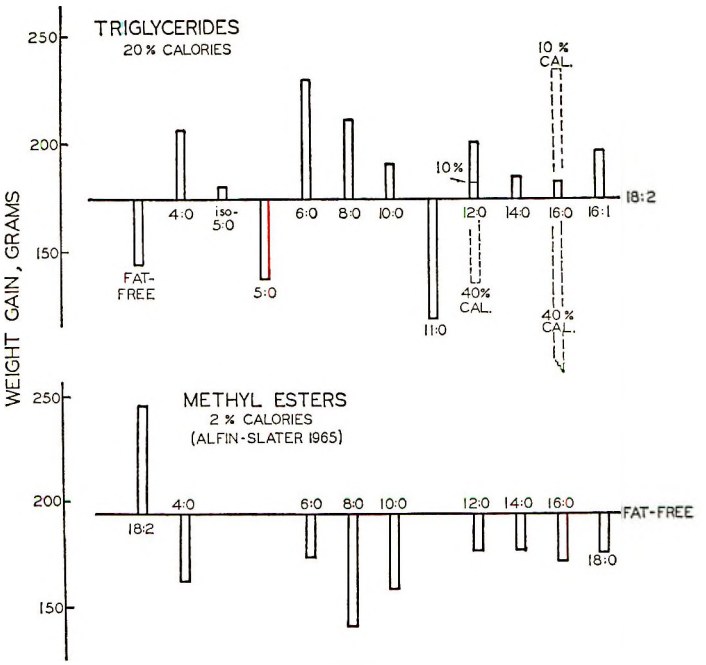

Fig. 1 *Upper half*: Effect of dietary triglycerides of saturated fatty acids upon weight gain of rats fed a supplement of linoleic acid. Horizontal line represents the weight gained by rats fed a fat-free diet *with* a supplement of ethyl linoleate. *Lower half:* Effect of dietary methyl esters of saturated fatty acids upon weight gain of rats fed a fat-free diet. Horizontal line represents the weight gained by rats fed a fat-free diet *without* a supplement of linoleic acid. The dietary triglycerides and methyl esters, respectively, are designated by the shorthand formula of their constituent fatty acid, i.e., tributyrin, 4:0.

alone, feeding of the same triglycerides at 40% of calories had the contrary effect which was very pronounced for tripalmitin. Rats fed tripalmitin at 40% of calories remained at their original weight of 50 g with food intakes one-third as high as the other groups. In this group, 4 out of 6 animals died as compared with no significant mortality in the others. Animals fed trilaurin at 10% of calories did not show a significant difference from those fed trilaurin at 20% of calories, whereas tripalmitin fed at 10% of calories supported better weight gain than it did at 20% of calories.

The lower part of figure 1 shows weight gain of rats which received supplements of saturated fatty acids to an otherwise fat-free diet as reported recently by Alfin-Slater and co-workers (14). Addition of saturated fatty acids to a fat-free diet resulted in lower weight gain. Comparison of these results with ours and consideration of the facts reported heretofore on the effect of dietary saturated fatty acids (6–13) upon growth of rats lead to these conclusions: 1) Rats maintained with a rigidly fat-free diet are unable to utilize dietary saturated fatty acids for growth. The saturated fatty acids, in fact, hinder growth compared with fat-free diets. 2) Growth of rats is stimulated if minimal quantities of linoleic acid are fed simultaneously with even-numbered saturated fatty acids. The growth rate exceeds that of rats fed fat-free diets and even of those fed the control level of linoleic acid. Short- and medium-chain length fatty acids appear to be more effective than long-chain fatty acids in stimulating growth. Saturated fatty acids fed in excess of 20% of calories have an adverse effect upon growth.

Fatty acid composition of liver lipids. Accumulation of fatty acids of chain lengths up to C_{10} was not observed in liver lipids of rats. This is most likely due to the analytical methods used. However, a significant deposition of saturated fatty acids of chain length C_{11} through C_{16} was observed in the liver lipids of rats supplied with the respective triglyceride in the diet.

Changes in the metabolism of linoleic acid are indicated most strongly by alterations of the concentration of 20:3ω9 [6] and 20:4ω6 in liver lipids. The former acid is increased by synthesis from 18:1ω9 if the conversion of linoleic to arachidonic acid is hindered by lack of substrate or by interaction of other fatty acids. Arachidonic acid is the predominant metabolite of 18:2ω6; its concentration will decrease if the conversion of linoleic to arachidonic acid is inhibited. Figure 2 shows that the levels of arachidonic acid in all rats fed triglycerides of saturated fatty acids were increased at least 50%, in most cases 100%, compared with those of rats maintained with the fat-free diet. In animals fed saturated triglycerides at 20% of cal-

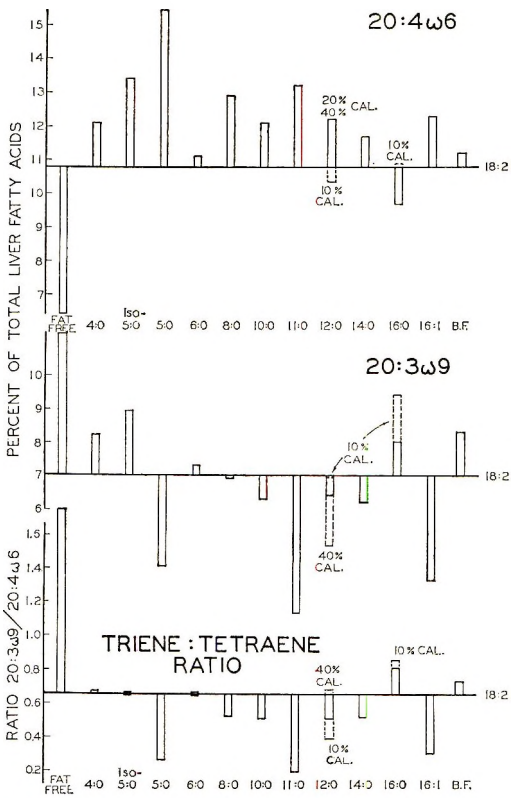

Fig. 2 Effects of dietary triglycerides of saturated fatty acids upon 20:4ω6, 20:3ω9 and the triene-to-tetraene ratio in liver lipids of rats fed a supplement of linoleic acid. Base lines represent the respective values when linoleate is fed as the *only* supplement. The dietary triglycerides are designated by the shorthand formula of their constituent fatty acid, i.e., tributyrin, 4:0; B.F. stands for synthetic butterfat.

[6] See footnote 3.

ories, arachidonic acid in liver lipids was even higher than in the control group fed linoleic acid only, with the exception of dietary tripalmitin which depressed the level of 20:4ω6 somewhat. This suggests that the growth-promoting effects of the even-numbered saturated fatty acids in the presence of dietary linoleic acid may be due to sparing of linoleic acid from β-oxidation. The highest levels of 20:4ω6 in liver lipids occurred in rats that grew least, namely those fed triglycerides of odd-chain saturated fatty acid. This indicates that these animals were not required to use their reserves of EFA for growth of new cells. The concentrations of the other metabolites of 18:2ω6, namely, 20:3ω6, 22:4ω6 and 22:5ω6, were elevated also but less significantly than that of 20:4ω6.

The 2 rats that survived feeding of a diet containing tripalmitin at 40% of calories differed widely in the fatty acid composition of their liver lipids. Whereas one animal was able to convert the dietary 18:2ω6 and accumulate or preserve as high a level of arachidonate as 22% of the total fatty acids, the other one had a level of arachidonic acid in the same order of magnitude as fat-deficient animals. Since the number of animals involved is too small, conclusions cannot be drawn and related data were not included in figure 2.

The levels of 20:3ω9 of all animals fed saturated acids were significantly lower than in rats fed the fat-free diet (fig. 2). In fact, triglycerides of all fatty acids but 4:0, iso-5:0 and 16:0 supported levels of 20:3ω9 equal to or lower than that of the control animals fed linoleic acid only. It should be noted at this point that the linoleic acid level fed did not meet the requirement of the rats. Thus, a substantial amount of 20:3ω9 was synthesized even in the rats supplemented with linoleic acid. The lowest concentration of 20:3ω9 occurred in the liver lipids of groups fed triglycerides of 5:0, 11:0, and 40% of calories of 12:0. These rats did not grow as well as the others. Dietary tributyrin, tri-iso-valerin and tripalmitin, although maintaining growth rates better than dietary linoleic acid alone and enhancing the synthesis of 20:4ω6, produced higher levels of 20:3ω9 than those observed for the controls.

The combined effects of lowering the level of 20:3ω9 and raising the concentration of 20:4ω6 are demonstrated by plotting the triene-to-tetraene ratio, which has been used to assess metabolic status with respect to EFA deficiency (15). The triene-to-tetraene ratio which was 0.66 in the linoleic acid-supplemented group was maintained or even lowered by all the dietary triglycerides under investigation, except that of 16:0 which increased it significantly. Therefore, we conclude that even-numbered saturated fatty acids up through C_{16} do not inhibit the conversion of linoleic to arachidonic acid if minimal amounts of linoleic acid are provided in the diet.

The odd-numbered fatty acids, however, lowered the triene-to-tetraene ratio beyond 0.4 which is usually considered an indication for sufficient EFA, the consequence of inhibited growth.

Effect of dietary palmitoleic acid upon the metabolism of linoleic acid. Palmitoleic acid fed as triglyceride did not interfere with growth of rats given the linoleic acid supplement. Average weight gain of rats fed 20% of calories of 16:1ω7 was significantly higher than that of the control group fed linoleic acid (fig. 1). The deposition of dietary palmitoleic acid in liver lipids was clearly indicated by an increase of this acid from an average of 7% to 20% of the total fatty acids.

The level of 20:4ω6 in liver lipids of rats fed tripalmitolein was higher than in animals fed linoleic acid only, indicating that this dietary monoenoic fatty acid does not interfere with the conversion of linoleic acid to arachidonic acid (fig. 2). The content of 20:3ω9 of liver lipids was lowered most markedly by dietary tripalmitolein which might be due to a sparing of 18:2ω6 from β-oxidation and the contribution of unsaturation to lipids by the deposition and further conversions of 16:1ω7.

The triene-to-tetraene ratio of liver fatty acids from rats fed tripalmitolein was lower than that of the control animals fed linoleic acid only. It was, in fact, lower than 0.4, a figure indicating sufficient supplementation of rats with essential fatty acids. This shows that tripalmitolein fed at the level of 20% of calories of the diet does not upset the normal conversions of

linoleic acid. The effects of 16:1 studied here are more closely related to those of dietary saturated fatty acids than to those of the monoenoic fatty acids of 18 carbon atoms (4).

Effect of "synthetic butterfat" upon the metabolism of linoleic acid. As butter sometimes contains less than the required amount of linoleic acid, but large amounts of the saturated fatty acids, the appropriate amounts of the triglycerides of fatty acids normally present in butterfat (16) were fed under the same conditions as were the individual triglycerides in this experiment. The average weight gain effected by the "butterfat," 204 g, was in the same range as that for dietary triglycerides of 10:0, 12:0 and 16:1, all being well above the weight gain of the rats fed linoleic acid only. The level of 20:4ω6 in liver lipids of animals fed "butterfat" was slightly higher than that of the control animals fed linoleic acid. The 20:3ω9 level and the triene-to-tetraene ratio were somewhat higher, however, in rats fed the "butterfat" than in the controls. This indicates that the combination of saturated and monoenoic acids contained in "butterfat" fed to rats does not significantly interfere with the metabolism of linoleic acid supplied in the diet.

Incorporation of odd-numbered saturated fatty acids in liver lipids. The effects of trivalerin and triundecanoin as dietary lipids upon the metabolism of linoleic acid have been described in the preceding paragraphs. In addition to these effects, a substantial increase of odd-numbered long-chain saturated and unsaturated fatty acids was observed in liver lipids when triglycerides of 5:0 and 11:0 were fed (table 2). The increases of long-chain odd-numbered fatty acids were significant, but concentration of these acids in liver lipids induced by dietary 5:0 and 11:0 were not the same.

Since the decrease in 20:3ω9 levels was most pronounced in liver lipids of rats fed the triglycerides of odd-numbered fatty acids, it is probable that the long-chain odd-numbered acids, especially the unsaturated acids 17:1 and 19:3, might have substituted for 20:3ω9 in liver lipids. The odd-numbered fatty acids are known to have lower melting points than the next higher even-numbered homologous fatty acids and, thus, they could, for instance, maintain a physical property of lipids that normally can only be supplied by long-chain polyunsaturated even-numbered fatty acids.

Polyunsaturated acids are predominantly located in the β-position of lecithins in rat liver. Therefore, we determined the position of the odd-numbered fatty acids in the lecithin fraction of the liver lipids from the rats fed triglycerides of 5:0 and 11:0. The lecithin was isolated from the lipids by column and thin-layer chromatography (18, 19). It was then incubated with phospholipase A [7] from *Crotalus adamanteus* venom (20). The fatty acid composition of the liberated fatty acids, presumably from the β-position of the lecithin, and from the remaining lysolecithin was determined by GLC.

The saturated odd-numbered fatty acids, 13:0, 15:0 and 17:0, were each represented in the β-position by less than 33% which is the same order as found for 16:0.

[7] Ross Allen's Reptile Institute, Silver Springs, Florida.

TABLE 2

Concentration of odd-numbered fatty acids in liver lipids

Fat supplement of diet [1]	Odd-numbered fatty acids in liver lipids				
	15:0	17:0	17:1	19:3	Total
	% of total fatty acids				
Triglycerides of even-numbered fatty acids [2]	0.2	0.1	0.3	0.4	1.0
Trivalerin	0.8	2.4	3.0	2.1	8.3
Triundecanoin	1.4	1.9	1.5	0.9	5.7

[1] See footnote 1, table 1.
[2] The average was computed for the long-chain fatty acids found in liver lipids of rats fed the triglycerides of 4:0, 6:0, 8:0, 10:0, 12:0, 14:0 and 16:0.

The 17:1 and 19:3, however, were each present in amounts near 50% in this position as compared with 65% for 20:4ω6. Thus, there is some indication that unsaturated odd-numbered fatty acids may be substituted for 20:3ω9 in the lecithin molecule in the β-position (21).

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A Probable Direct Role of Ethanol in the Pathogenesis of Fat Infiltration in the Rat [1]

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ABSTRACT Recently, a fat release mechanism was proposed that could explain, at least partially, fat infiltration resulting from carbon tetrachloride poisoning. Hence, a study was made to determine whether such a mechanism also operates subsequent to alcohol intoxication. Normal and chronically ethanol-treated rats were used after maintaining them with an adequate purified diet for 40 weeks. The ethanol-treated rats were given 20% alcohol as the sole drinking fluid. Either water or a 1:1 ethanol solution (95% ethanol:water, v/v) was given to the animals of both groups with a stomach tube and a fat-clearing blocking agent (Superinone) was injected intravenously 2 hours after intubation. Ninety minutes later blood plasma and livers were analyzed for triglyceride content. The results indicate that ethanol during both acute and chronic intoxication probably produces a direct toxic effect on the liver which could interfere with the release of hepatic triglycerides into the blood. Moreover, the transport of peripheral triglycerides to the liver does not appear to be a major factor in alcoholic fatty infiltration.

Previous studies (1, 2) have given some insight into the process of hepatic fat accumulation subsequent to carbon tetrachloride poisoning. More recently, however an hypothesis has been offered to explain the underlying biochemical alterations produced by this hepatotoxic substance which would lead to accumulation of fat in the liver (3). The hypothesis embraces the thesis that large amounts of triglycerides are continuously released from the liver and that carbon tetrachloride destroys this releasing mechanism, thus resulting in fat accumulation. Hepatic fat infiltration is likewise known to occur during acute and chronic alcohol intoxication. Whether ethanol induces a similar effect on the alleged triglyceride releasing mechanism in either or both types of intoxication is not clear. The purpose of the present study was to gain more insight into this hypothetical mechanism for consideration along with other mechanisms in the production of alcoholic fatty livers that have been suggested previously (4-7).

EXPERIMENTAL

Sixty-four male albino rats of the Sprague-Dawley strain, weighing 100 to 120 g, were fed for 5 weeks a purified diet containing adequate amounts of lipotropic factors and vitamins (8). The animals were divided equally into 2 groups of comparable weights. One group was given water and served as a control. The other group had access to 10% ethanol as the sole drinking fluid. After a 6-week period of adjustment, the concentration of alcohol was raised to 20% and kept at this level for the remainder of the 40-week experiment. Both diet and fluids were given ad libitum.

For the study of the effect of ethanol on the hepatic triglyceride release the procedure of Recknagel et al. (3), which was used for the study of the pathogenesis of carbon tetrachloride fat infiltration, was adopted and modified as follows: Sixteen normal and 16 alcohol-treated animals weighing from 400 to 500 g were used. The rats were fasted for 18 hours and each group was then divided into 4 equal subgroups. Two of these subgroups were force-fed water (1 ml/100 g body weight) with a stomach tube under light ether anesthesia and the other two were force-fed a 1:1 ethanol solution (95% ethanol:water, v/v), 1 ml/100 g body weight. Two hours later, 4 water-fed rats and 4 ethanol-fed rats were injected intravenously with 1 ml of 0.9% NaCl. Similarly, the other 8 rats

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of the group were injected with 1 ml of a 0.9% NaCl solution containing 100 mg of Superinone [2] [a brand of tyloxapol (oxyethylated tertiary octylphenol-formaldehyde polymer)]. Ninety minutes subsequent to these injections blood was withdrawn from the abdominal aorta and the livers were removed and weighed for triglyceride determinations. At the outset, because Superinone has been shown to affect only esterified lipids (11), plasma and hepatic triglyceride levels and not free fatty acids were measured in this study, although the latter might give more information under certain conditions. Nevertheless, it can be argued that the study of the influx and egress of esterified hepatic lipids is more informative for the present investigation since most of the lipids in fatty infiltration are thought to be in the esterified form. Plasma triglycerides were determined by the method of Van Handel and Zilversmit (9) and liver triglycerides were determined according to Butler and his collaborators (10). All experiments were made in duplicate.

RESULTS

The data in table 1 show clearly that force-feeding of ethanol to normal rats lowered the plasma triglyceride (PTG) level to almost two-thirds that of the control (rows A and C, $P < 0.001$).[3] Moreover, Superinone, which was shown by others to interfere seriously with the disappearance of both PTG (12) and plasma phospholipids (13) by possibly inhibiting the plasma lipid-clearing system (11), raised the PTG level tenfold in the water-fed subgroups (rows A and B, $P < 0.0001$) and only 7-fold in the ethanol-fed subgroups (rows C and D, $P < 0.001$). In contrast with the water-fed animals, Superinone raised the level of PTG fivefold in the presence of ethanol (rows A and D, $P < 0.001$). The results in table 1 indicate further that hepatic triglycerides (HTG) were doubled in the 3.5-hour interval after the administration of ethanol (rows A and C) in the absence of Superinone but decreased slightly in its presence (rows A and B and C and D).

These results suggest that ethanol appears to possess a toxic effect, probably a direct one, on the liver. Moreover, the transport of PTG from the periphery into the liver under the influence of ethanol intoxication may not be a major contributing factor in the alcoholic fatty infiltration. These points will be elaborated on in the discussion. In addition, one can speculate that Superinone probably does not interfere with either the biosynthetic functions of the liver, at least with respect to triglyceries, or with the release of these

[2] Superinone was kindly supplied by the Sterling-Winthrop Research Institute, Rensselaer, New York. It may be obtained also from the Rohm and Haas Company, Philadelphia, under the trade name Triton WR1339.

[3] Significant at $P < 0.05$ level.

TABLE 1

Effect of ethanol on plasma and hepatic triglyceride level of normal and alcohol-treated rats [1]

Subgroup	Treatment of animals	Plasma triglycerides (PTG)	Hepatic triglycerides (HTG)	
		mg/100 ml	*mg/g liver*	*mg/whole liver/100 g body wt*
		Group 1, normal rats		
A	Water	21.00 ± 2.19 [2]	6.00 ± 0.80	22.20 ± 1.90
B	Water + Superinone [3]	226.00 ± 2.63	5.60 ± 0.87	19.08 ± 1.80
C	Ethanol [4]	14.20 ± 0.665	12.30 ± 1.22	46.60 ± 3.35
D	Ethanol [4] + Superinone	105.00 ± 3.74	12.20 ± 1.20	43.40 ± 1.96
		Group 2, alcohol-treated rats		
E	Water	37.80 ± 2.71	9.82 ± 1.11	44.91 ± 2.63
F	Water + Superinone	345.06 ± 4.78	9.40 ± 1.20	42.60 ± 1.87
G	Ethanol [4]	28.40 ± 2.48	12.70 ± 1.30	49.80 ± 1.98
H	Ethanol [4] + Superinone	270.80 ± 6.88	11.54 ± 1.20	47.98 ± 2.58

[1] Eight rats/group, total number in duplicate experiments.

[2] Average ± SD.

[3] A brand of tyloxapol (oxyethylated tertiary octylphenol-formaldehyde polymer), supplied by the Sterling-Winthrop Research Institute, Rensselaer, New York.

[4] 95% ethanol:water (1:1, v/v).

fats into the plasma. This speculation is based on the reported mechanism of action of Superinone (11) as well as on the observed large increase in the level of PTG in its presence since PTG is believed to originate mostly from the liver. A definitive answer to this question should await further investgation.

In the alcohol-treated group the results were, in general, quantitatively and to a certain extent qualitatively, different from those obtained in the nontreated rats. Force-feeding of ethanol apparently lowered the level of PTG to only three-fourths that of the control (rows E and G, $P < 0.01$). However, when Superinone was later injected the triglycerides increased tenfold (rows G and H, $P < 0.001$), 1.5 times higher than in the corresponding subgroups of the normal animals (rows C and D). Superinone in the absence of ethanol, on the other hand, caused a ninefold increase in PTG (rows E and F, $P < 0.0001$) which is comparable to the corresponding values for the normal rats (rows A and B). Moreover, the PTG level was consistently 1.5 to 2.5 times higher in the alcohol-treated rats than in the normal animals irrespective of whether Superinone was administered (rows A and E; B and F; C and G; D and H).

With respect to HTG, all subsequent discussion refers to data for whole liver per 100 g of body weight, although the values per gram of liver gave essentially similar results. Prolonged treatment with alcohol appeared to have caused an approximately twofold accumulation in HTG as compared with normal rats (rows A and E, $P < 0.001$). Force-feeding of ethanol did not substantially increase this level further (nearly 12%, rows G and H as compared with E and F, respectively, $P > 0.05$), whereas in the normal rats it was doubled (rows C and D in contrast with A and B). As in the normal rats there was a slight change in the HTG of the alcohol-treated animals subsequent to Superinone treatment.

The results of the experiments on the alcohol-treated rats support the thesis that ethanol could have an inhibitory effect on the release of triglycerides from the liver. The data, in addition, agree with the results of experiments performed on the normal rats with respect to the transport of fat from plasma into the liver under the influence of alcohol intoxication, although they differ in the extent of operation of this process. Furthermore, the results with Superinone indicate that this agent has effects comparable to those observed in normal rats.

DISCUSSION

Ethanol could play at least three important roles in the accumulation and transport of hepatic lipids, namely, enhancing fat synthesis in situ, increasing transport of lipids from the periphery into the liver, and possibly affecting their release from the liver into plasma. Stimulation of synthesis has been shown previously to be a major contributor to the accumulation of hepatic fat (8, 15). However, a normal or increased transport of fat from the periphery into the liver has not yet been established as significant. Nevertheless, Brodie and co-workers (7) have presented evidence for an alcohol-induced triglyceride deposition in liver through derangement of fat transport. Although the results point to an actual transport, their data do not show how much this alteration contributes to the process of hepatic fat accumulation. Likewise, the data of the present study do not show that this transport mechanism is a major factor during both acute or chronic alcohol intoxication. In fact only a small portion of hepatic lipids could have originated from the periphery. This is clear because liver triglycerides did not decrease in this investigation to more than 12% with Superinone at a time when the plasma triglycerides increased tenfold in the control and fivefold in the alcohol-treated rats. Had the major source of hepatic triglycerides been from the periphery, the decrease would be expected to be even more in the presence of this inhibitor than that actually observed, probably commensurating with the actual rise in the level of PTG under the conditions of the experiment. It can be argued that a greater decrease in HTG could have actually occurred but was offset, at least partially, by an accumulation of fat due to other mechanisms, as for instance, an enhanced synthesis in situ, a decreased hepatic oxidation, a decreased release from the liver, or to some other

factors not yet measured. Further work is needed to clarify this point.

Despite the stimulating effect of ethanol on the synthesis of hepatic fat mentioned above, the PTG level in the ethanol-fed rats after Superinone injection was raised to only half the corresponding level of the control animals. Even in the absence of Superinone the level was nearly two-thirds that of the control. A similar effect of alcohol was observed in the alcohol-treated rats but to a lesser extent. Statistical evaluation of these differences has confirmed their significance ($P < 0.05$). These observations can be explained by the premise that alcohol could have caused a derangement in the fat-releasing mechanism of the liver. This would appear tenable if the liver is the main source of plasma triglycerides. In fact, Byers and Friedman (12) have presented conclusive evidence that PTG's originate primarily from the liver and that the gastrointestinal system plays a relatively minor role, with that of the other fat depots being even negligible.

Data on the liver appear to lend further support to the concept of impaired release mechanism of hepatic fat. Ethanol feeding to normal rats doubled the level of triglycerides (rows C and D as compared with A and B). In addition to an interference with the release of triglycerides, however, this increase could be attributed, at least in part, to a decrease in oxidation of hepatic fat or to actual de novo synthesis. In fact alcohol intoxication was shown to decrease fat oxidation in in vivo and in vitro experiments, although only slightly (16-19). Likewise, enhanced fat synthesis under conditions similar to those of the present experiment was also reported (15, 17, 19). However, it is not conceivable that hepatic triglycerides would be doubled in such a short period of acute intoxication if an intact releasing mechanism was operating simultaneously. Normally, in the absence of intoxicating agents, the exit of fat is believed to occur very rapidly (14). In this connection it is believed also that the transport of fat is generally in the direction from the liver to adipose depots (20). On the basis of the present experiment it would not be unreasonable to assume that a great portion of the observed doubling in the triglyceride content of the liver is primarily a reflection of a direct toxic effect of ethanol on the release mechanism similar to that proposed for carbon tetrachloride (1, 2). The hepato-toxicity of ethanol, in contrast with CCl_4 would not be as pronounced and its action as rapid. This is evident from the present study as well as from the report of Recknagel et al. (3).

In the alcohol-treated rats, however, the increase in hepatic triglycerides was not as pronounced as in the normal group. In this case the rise was only 12% (rows G and H as compared with E and F, respectively). Statistically, this rise is only slightly insignificant ($P = 0.07$). However, that ethanol caused a significant increase in HTG of the normal group in the presence and absence of Superinone favors the theory for retardation of the proposed release mechanism by alcohol. The apparent differences between the 2 groups should be explained on different grounds, however. Attention should be drawn to the fact that here the level of HTG was already high at the start of the determinations (nearly double that of the normal rats). This is not unexpected since chronic ingestion of ethanol could cause excessive synthesis of fat and induce a slower rate of its oxidation. Also, it may be that the difference in increment of HTG in both groups is due to an alteration in the release mechanism that had already occurred during prolonged alcohol consumption. Nevertheless, these observations suggest a common mechanism for the effect of ethanol in both groups and perhaps for other active factors conducive to fat infiltration yet to be determined.

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The Porcupine Cecal Fermentation [1]

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ABSTRACT The fermentation in the cecum of the wild porcupine was studied to determine the principle products, their rates of formation, percentage absorption and contribution toward satisfying the energy requirement of the animal. Fermentation rates were determined by the zero-time method using cecal content from slaughtered animals. Volatile fatty acids (VFA) were determined by gas chromatography. VFA levels in the cecal blood were also measured. The fermentation resembled that of a ruminant eating a high roughage diet. The products were chiefly the VFA acetic, propionic and butyric, found in average proportions of 74, 12 and 14% respectively. Most of these were absorbed with 88% of the absorption from the cecum and 12% from the large intestine. Absorption rates are proportional to concentrations. The average contribution of the cecal fermentation to the maintenance energy requirements of the animal was 16% but was as high as 33% in one animal.

Herbivorous animals are characterized by having one or more expanded portions in their alimentary tract besides the stomach. Ruminant animals have a rumen, reticulum and an omasum cepahalic to the true stomach. The cecum is large in most rodents and in some the stomach has a proventriculum. The horse and elephant are examples of animals with a large cecum and colon. These expanded portions of the digestive tract are usually sites of active fermentations that aid in food digestion and produce fermentation products useful to the animal.

These products have been shown by several workers, Elsden (1), Elsden et al. (2) and Hungate et al. (3), to be chiefly the short-chain volatile fatty acids (VFA). Their average proportions in the alimentary tracts of sheep, red deer, rabbits, rats and pigs were reported by Elsden et al. (2) to be 67% acetic, 19% propionic and 14% butyric. Variations may occur with diet.

The VFA's are absorbed into the blood of the animal. Barcroft et al. (4) observed the blood draining the rumen and cecum of sheep and the cecum, colon, or both, of the horse, pig and rabbit to have a higher concentration of the VFA's than peripheral blood. The contribution of fermentation products to the energy requirements of the animal has been estimated at from 3 to 70% for cattle, Carroll and Hungate (5), Emery et al. (6), Stewart et al. (7) and Hungate et al. (8), and 50% for deer, Short (9).

This investigation was concerned with a better understanding of the importance of the cecal fermentation to the nutrition of a nonruminant animal having a large cecum. The yellow-haired porcupine (*Eretizon dorsatum epixanthum*) was selected as the experimental animal because it is a large rodent, thus providing adequate quantities of cecal material for a variety of analyses; is readily available in this area; can be collected without special permits; and is relatively easy to catch. The porcupine is also of interest because its diet may include materials difficult to digest, such as bark and the cambium layer of conifer and deciduous trees as well as succulent plants. It is also important that the nutrition of the porcupine is not complicated by coprophagy.

METHODS

The porcupines were caught by local ranchers usually along the wooded edges of hay meadows and grain fields. Experiments using wild animals were performed within 12 hours of capture and hence the digestive tracts contained the natural foods. Withdrawal of arterial and venous blood from the cecal vessels after ether

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anesthesia and collection of digestive system contents following chloroform-induced death was generally made from each animal. The alimentary tract was removed; the stomach and cecum were weighed, and the lengths of the intestines measured and the contents of all these organs examined.

The contents of the large intestine were divided into 3 portions; LI_1, the first 25 cm caudal to the cecum, LI_2, the next 25 cm section, and LI_3, the remainder containing the fecal pellets in varying states of dehydration. The percentage of dry matter was determined by drying aliquot samples at 80° to constant weight. Portions of the contents from the stomach, small intestine, cecum and the 3 sections of the large intestine were stabilized by mixing 1:1 (w/v) with 0.2 N H_2SO_4, centrifuged and the clear supernatent saved for chemical analysis.

Rates of VFA production in the cecum were determined by the zero-time method of Carroll and Hungate (5). Cecal contents were incubated at 39° in an atmosphere of CO_2. The first sample was inactivated at the time of transfer into the incubation flask; subsequent samples were removed at hourly intervals. Total VFA's were determined by steam distillation (10) of 1- to 5-ml aliquots of the acidified samples. Fifteen volumes of distillate were collected and titrated with 0.02 N NaOH to the bromthymol blue endpoint while being mixed with a stream of CO_2 free air. The individual VFA's were identified and determined quantitatively by gas-liquid chromatography. The acids from 10 to 20 ml of the acidified sample were collected by steam distillation, made alkaline to thymol blue with NaOH (pH 9.6 or higher) and dried on a steam bath. The dried salts were dissolved in 0.2 ml of hot distilled water, chilled in an ice bath and acidified with 2 or 3 drops of concentrated H_2SO_4. The VFA's were extracted by rapidly mixing the solution with 5 ml of dichloromethane and adding 2 g of anhydrous Na_2SO_4 to remove the water. The mixture and rinsings were poured onto a medium-porosity fritted glass filter and the solvent was collected in a 10-ml volumetric flask. The volumetric flask was filled to volume with dichloromethane just prior to analysis. This method was found to extract acetic and higher VFA's quantitatively from the water solution.

Thirty-microliter samples were injected into a Beckman GC 2A gas chromatograph. The packing in a 6.25-mm stainless steel column 180 cm in length was a modification of that used by Erwin et al. (11); Gas Chrom P (60–80 mesh) coated with 0.74% H_3PO_4 and 7.4% polyoxyethylene sorbitan monooleate (Tween 80). The column was operated at 130°, a helium flow of 110 ml/minute and a thermal detector current of 300 ma. The output was recorded on a Texas Instruments Servo-Riter integrating recorder.

Standards of reagent grade acetic, propionic and butyric acids dissolved in dichloromethane gave linear results between 0.003 and 0.100 mEq/ml when using a 30-uliter sample. An equation for the standard curve of each acid was calculated by the method of least squares.

RESULTS

General observations. Since we were interested in the importance of the cecal fermentation in the nutrition of the porcupine, it was desirable to study wild animals eating their natural food. Catching animals not influenced by man and his agriculture proved to be difficult; more than half were caught in alfalfa or wheat fields. Porcupines are nocturnal and were usually caught while feeding between the hours of 7 and 10 PM. Twenty-seven wild animals collected between February, 1961, and October, 1963, were examined.

The cecum is caudal to the small intestine and in the absence of adequate data on the turnover rate of its contents, the amount of cecal content might be expected to be relatively more constant than that of the rumen; but this did not appear to be true. The weight of the cecum with its contents varies from 2.8 to 10% of the total body weight (table 1). The average was 5.97%. This is greater than the relative weight of the horse cecum (2.5%) but less than that of the rabbit (7.8%) and rat (10.5%) as reported by Elsden et al. (2) It should be noted that the large intestine is generally longer than the small intestine. This length of the large intestine suggests its importance for the absorption of cecal fermentation products.

TABLE 1

Weights and measurements of animals and organs

	Body wt (27) [1]	Stomach (26)		Full cecum (26)		Empty cecum (5)		Small intestine (7)	Large intestine (7)
	kg	*g*	*% of body wt*	*g*	*% of body wt*	*g*	*% of body wt*	*cm*	*cm*
Range	3.0–13.6	117–628	1.8–8.2	244–712	2.8–10.0	50–120	1.3–1.8	160–280	230–280
Average	8.25	304	4.1	437	6.0	77.2	1.57	223	252
SE of mean	±0.59	±24	±0.4	±8	±0.4	±12.3	±0.01	±16	±7

[1] Number of animals/group.

This function is confirmed by the observation that the cecum contained 0.511 mEq VFA/g dry weight where LI_3 contained 0.183 mEq, a 64% reduction (table 2). The dry material content of these 2 fractions was 15% and 34%, respectively, a 22% reduction in water content.

The minimal rate of food passage through the cecum, assuming no loss of solids in the large intestine, was taken to be the same as the dry weight of feces excreted per day. This was found to be 9.4 ± 0.6 [3] g/day/kg of animal weight for porcupines fed antibiotic-free rabbit pellets. Under natural conditions this amount would vary with the diet.

The validity of samples taken from cecal content is dependent upon the uniformity of the contents. Since the ileum is more alkaline (above pH 8) than the cecum, the pH of the cecal contents was used as an index of mixing. Measurements on 14 cecums gave average pH values of 6.44 ± 0.06 within 3 cm of the ileocecal valve and 6.25 ± 0.22 at the closed tip. Mixing in the porcupine cecum was observed by placing a $BaSO_4$ suspension in the cecum of a restrained cannulated animal and watching its rate of dispersion with a fluoroscope. The $BaSO_4$ was uniformly dispersed throughout the cecum in about 20 minutes. The mixing of the cecal content may be more rapid in a normal animal since the cannula may restrict the cecal movement. In a similar experiment $BaSO_4$ added into the stomach of a rabbit with a stomach cannula was intimately mixed throughout the cecum in less than a minute after entering from the small intestine.

Fermentation products. The only detectable non-gaseous fermentation products were acetic, propionic and butyric acids. No other VFA's were present in sufficient quantity to be qualitatively shown by gas chromatography. Thin-layer chromatography of the residue following steam distillation did not show any lactic or succinic acids and ethanol was not present in a distillate from acidified cecal content following a redistillation from an alkaline solution and testing for volatile reducing materials with a chromic acid solution. The total VFA in the ingesta was determined for 25 animals (table 2). Most

[3] Average ± SE of mean.

of the VFA's are produced in the cecum, very little, if any, being formed in the stomach or small intestine. The concentrations of VFA found in the stomach, cecum and LI_3 are similar to those reported by Elsden et al. (2) in the stomach, cecum and rectum of the horse, rabbit and rat. The difference between the VFA concentrations of the cecum and LI_3 shows that about 64% of the acids leaving the cecum are absorbed from the upper half of the large intestine. Total acid absorption was not measured, but could be greater than this amount if acid absorption continues after fecal pellet formation begins. The acids are absorbed preferentially to water. There is no indication, however, of preferential absorption of any one of the 3 acids measured (table 3).

The production rates of the individual VFA in the cecum at the time of death of 11 animals are shown in table 4. Acetic acid production was lower in animals kept without food for 12 hours, whereas propionic and butyric acid production was affected little if at all by the time lag (animals P25 and P27). Cecal VFA concentration in these animals varied directly with the rate of acid production, but in the large intestine the rates of production were decreased and the acids were absorbed to consistently low levels. The ratios of the acids remained relatively constant during a 4-hour in vitro incubation of cecal content (table 5), indicating that VFA had been absorbed from the cecum in the same ratios as they were produced.

The concentrations of VFA in the cecal blood of 13 porcupines were found to average 6.01 ± 0.64 mEq/liter of venous and 4.00 ± 0.35 mEq/liter of arterial blood. The differences in concentration between the venous and arterial blood (V-A) were compared with those reported for the portal and carotid blood (P-C) of sheep by Schambye and Phillipson (11) and Schambye (12). The average V-A values were 23% higher than the P-C values for sheep (2.01 ± 0.53 and 1.64 mEq/liter); the maximum was much higher (6.21 mEq/liter for the porcupine vs. 2.7 mEq/liter) for the sheep. There is active absorption of VFA from the porcupine cecum and the blood levels of the porcupine are comparable to those observed in the ruminant. Whether the rate of blood flow and consequently the rate of absorption varies with the fermentation rates as in the ruminant (14) has not been determined; neither has the problem of acid metabolism while passing through the cecal wall been studied. The rumen and cecal walls are very different and hence the rumen results are probably not applicable.

TABLE 2

Volatile fatty acid (VFA) concentration in digestive organ content

	Organ				
	Stomach (23) [1]	Cecum (25)	LI_1 (23)	LI_2 (24)	LI_3 (24)
	mEq VFA/g dry wt of ingesta				
Range	0.017–0.172	0.278–1.33	0.174–0.893	0.145–0.493	0.113–0.292
Average	0.089	0.511	0.389	0.286	0.183
SE of mean	±0.012	±0.049	±0.039	±0.021	±0.01

[1] Number of animals/group.

TABLE 3

Composition of cecal and large intestinal volatile fatty acids

Organ	No. of animals	Acetic	Propionic	Butyric
		molar %	*molar %*	*molar %*
Cecum	11	74.2±2.0 [1]	12.0±0.8	13.8±0.9
LI_1	7	73.3±1.8	12.5±1.0	1–.1±1.0
LI_2	5	70.0±1.5	14.8±1.1	15.2±0.4
LI_3	7	73.5±2.6	13.8±1.3	12.6±1.5

[1] Averages ± SE of mean.

TABLE 4

Cecal volatile fatty acid production

Animal no.	Time, capture to experiment	Acetic	Propionic	Butyric
	hr		*mEq/g dry wt/hr*	
P 22	1	0.075	0.015	0.015
P 23	1	0.100	0.015	0.016
P 24	1	0.210	0.015	0.015
P 25	12	0.030	0.020	0.010
P 26	2	0.225	0.010	0.025
P 27	12	0.045	0.020	0.005
P 30	2	0.300	0.037	0.037
P 31	1	0.125	0.017	0.030
P 32	5	0.105	0.025	0.020
P 33	2	0.112	0.020	0.020
P 34	5	0.260	0.030	0.025
		0.144 ± 0.099 [1]	0.020 ± 0.002	0.020 ± 0.002

[1] Averages ± SE of mean.

TABLE 5

Composiiton of cecal volatile fatty acids during in vitro fermentation rate studies

Incubation time	No. of experiments	Acetic	Propionic	Butyric
hr		*molar %*	*molar %*	*molar %*
0.5	11	74.25 ± 1.19 [1]	11.98 ± 0.81	13.78 ± 2.83
1	9	73.96 ± 0.95	12.22 ± 0.78	14.17 ± 0.73
2	9	72.80 ± 0.33	13.14 ± 0.71	12.92 ± 2.11
3	6	70.77 ± 1.03	13.90 ± 0.82	15.38 ± 0.77
4	4	72.45 ± 1.52	11.75 ± 1.50	15.70 ± 1.07

[1] Averages ± SE of mean.

DISCUSSION

There is an active fermentation in the porcupine cecum and the VFA's produced there are largely absorbed by the animal. These acids averaged 74% acetic, 12% propionic and 14% butyric. The ratios are similar to those noted in the bovine rumen when the animal is eating a high roughage diet,[4] or in sheep following 24 hours without feed.[5] This is not unexpected when considering that the substrates available to the cecal bacteria are the residues following digestion by the animal, the equivalent of roughage.

The data show that an animal without feed for 5 hours (P32) had no decrease in the fermentation rate from that expected, whereas 12 hours without eating resulted in a fermentation rate lower than average, P25 and P27. If a maximal fermentation rate is to be maintained in the cecum, the porcupine must eat at less than 12-hour intervals. Porcupines, being nocturnal, probably eat during much of the night; this could not be observed since few animals were caught after 10 PM. This time limit was set by the collectors, not by the animals. The difficulty in finding animals during the day made it impossible to study the cecal fermentation rate of wild animals during the daytime.

The average fermentation rates in 11 wild animals were acetic 0.144, propionic 0.020 and butyric 0.020 mEq/g dry weight/hour (table 4). A delay of 12 hours without food between capture and examination markedly reduced the rate of acetic acid formation but had little effect upon the production rates of propionic and butyric acids. If it is assumed that a wild animal eats at intervals of 6 to 8 hours, the highest fermentation rates observed may be close to the average under natural conditions. From the fermentation rate and the cecal volume an average fermentation yield for an average animal can be calcul-

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ated if we assume a constant fermentation rate. The average cecal content was 15% dry matter and made up 4.4% of the weight of the animal if the weight of the empty cecum is subtracted (table 1). Therefore a 10-kg porcupine would contain 66 g of dry cecal content. This amount of material would yield daily an average of 230 mEq acetic, 32 mEq propionic and 32 mEq butyric acid. Of these, 192 mEq acetic, 25.9 mEq propionic and 24.9 mEq butyric acids would be absorbed from the cecum and approximately 37.0 mEq acetic, 6.1 mEq of propionic and 7.1 mEq of butyric would pass to the large intestine with the approximately 100 g dry weight of material passing through the animal daily. Of the acids leaving the cecum, 64% are absorbed from the large intestine, or 24.4 mEq acetic, 3.6 mEq propionic and 4.8 mEq butyric per 24 hours. The daily total of acids absorbed would be 216 mEq acetic, 29.5 mEq propionic and 29.7 mEq butyric, the equivalent of 71 kcal of energy based on their heats of combustion. If it is assumed that the maintenance energy requirement is proportional to the three-fourths power of the body weight (15) and calculations are made from Kleiber's figures for other animals, the maintenance energy of a 10-kg porcupine would be 435 kcal/day. The products absorbed from the average cecal fermentation would thus provide about 16% of the required maintenance energy. The same type of calculation applied to the maximal fermentation rates and acid concentrations in the cecum, animal P30 (table 4), and the average values for absorption from the large intestine gives a value of 143 kcal, about 33% of the maintenance energy. Similarly the minimal fermentation rate (animal P25), yields 5.5% of the maintenance energy. The maintenance energy requirement of the porcupine is high because of its relatively small body size; 43.5 kcal/kg and 52.5 kcal/kg for 10-kg and 5-kg animals, respectively, as compared with 15.7 kcal/kg for a 500-kg bovine.

Studies on the individual volatile acids of the rumen have indicated that the absorption rates of different acids are not the same and that the concentrations of the individual acids may not depict the rates at which they are produced (4, 16, 17). If the rates of absorption are dependent not only upon concentration but also upon acid species, it would be expected that the ratios of the acids would change during the course of fermentation in vitro. This was not found to occur. This dependence of the absorption rate upon the concentration of the acids in the ingesta was substantiated by the nearly constant ratios of the acids in the 3 segments of the large intestine. This situation makes the study of a cecal fermentation less complex than that of the rumen fermentation.

About 83% of the VFA's produced in the cecum are absorbed into the blood directly from the cecum. Sixty-four per cent of the volatile acids which entered the large intestine were absorbed there. Therefore, of the volatile acids absorbed by the animal, about 88% were absorbed from the cecum and only 12% from the large intestine. The levels of VFA in the venous blood of the cecum also indicated a high rate of absorption from the cecum.

The porcupine is rarely, if ever, a coprophagous animal, and hence the crop of microorganisms grown in the cecum is excreted and is of no value nutritionally. The ruminant has the advantage of being able to use some of the organisms responsible for the rumen fermentation (8). Coprophagous rodents such as the rabbit also have this advantage to an unknown degree.

The porcupine cecal fermentation appears to be of increasing importance in larger animals. This could be offset, however, by younger animals having a proportionately larger cecum. There were not enough animals available to permit valid observations on this point and since a porcupine gives birth to but a single young a year it was not feasible to make a laboratory study of relative cecum size with age of the animal. It is also possible that the cecal fermentation is of the most importance during periods of poorest diet such as when deep snow covers all food except tree bark.

ACKNOWLEDGMENTS

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Elimination of Fixed Selenium by the Rat [1,2]

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ABSTRACT Studies were conducted to determine the retention of a single injected dose of selenite by rats, and efforts were made to alter the subsequent rate of excretion of retained selenium by dietary modifications. After a single subacute injection of labeled selenite, selenium was eliminated rapidly for about 3 days, and after a period of transition a slow constant rate of loss persisted for months. The amounts of labeled selenium retained after the rapid elimination phase varied with the size of the dose, but the rate of loss thereafter did not. A series of diets fed to rats after the fixed pool had been established showed the rate of depletion from that pool to vary with selenium intake during the depletion period; loss of ^{75}Se was unaffected by changes in diet 1) which hasten selenium deficiency (Torula yeast); 2) which increase volatilization (high protein and methionine, or a laboratory diet); or 3) which diminish toxicity (linseed meal) except as these diets varied in selenium content.

Whether selenium is administered by mouth or by injection, it is rapidly attached to body proteins (1–5), but unless the dose is very small, most is nevertheless eliminated from the animal within 3 days (1–10). The elimination of the remaining "fixed selenium" is a much slower process, and can extend over months. Previous studies from this laboratory have dealt with factors that alter the rapid elimination of selenium in urine (6,7), bile,[4] or expired air (6–8). The present report describes attempts to alter the process by which fixed selenium is slowly eliminated from the body of the rat.

METHODS

Male weanling rats of the Holtzman strain were distributed at random into groups of four or five and placed in individual galvanized wire cages. After a 2-week growth period with an appropriate diet, the rats were injected subcutaneously with labeled selenite which was diluted with stable selenite to provide the appropriate level of selenium and continued with the diet as before. Thereafter, each rat was placed periodically into an Armac whole-body liquid scintillation counter, and the radioactivity retained by the animal was measured. A suitable aliquot of the dose solution was used as a standard and was diluted to a volume similar to that of the rat. The radioactivity of the rat was compared with that of the standard after correction for coincidence losses and background, and the result was expressed as the percentage of the original dose remaining in the animal.

The diets consisted of a commercial rat ration,[5] a purified basal diet, or a diet based on Torula yeast. The purified basal diet contained the following: (in per cent) sucrose, 70.7; casein, 20; corn oil, 5; mineral premix,[6] 4.0; B-vitamin premix,[7] 0.2; and choline chloride, 0.1. The Torula diet contained the following: (in per cent) sucrose, 45.2; Torula yeast,[8] 40;

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[2] Ancillary experiments and further experimental details are recorded in a Ph.D. Thesis entitled "Metabolism of selenium in the rat and lamb." R. C. Ewan. University of Wisconsin, 1966.

[3] Present address: Department of Animal Science, Iowa State University, Ames, Iowa.

[4] Levander, O. A. 1965 Studies on the distribution of selenium in rats given arsenic. Ph.D. Thesis, University of Wisconsin, Madison, Wisconsin.

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

[6] The mineral premix contained the following: (in per cent) NaCl, 11.03; KCl, 12.60; KH_2PO_4, 32.56; $Ca_3(PO_4)_2$, 15.56; MgO, 3.17; $FePO_4$, 2.86; MnO_2, 0.012; $CuCl_2 \cdot 5H_2O$, 0.028; and KI, 0.005.

[7] B-vitamin premix contained the following: (in per cent) inositol, 10.00; Ca pantothenate, 2.00; nicotinic acid, 1.00; menadione, 0.40; riboflavin, 0.30; pyridoxine·HCl, 0.25; thiamine·HCl, 0.20; folic acid, 0.02; D-biotin, 0.01; vitamin B_{12} (0.1% trituration), 1.00; and sucrose, 84.82.

[8] USP grade, Lake States Yeast Corporation, Rhinelander, Wisconsin.

stripped lard,[9] 10.0; mineral premix,[10] 4.0; DL-methionine, 0.5; B-vitamin premix,[11] 0.2; and choline chloride, 0.1. Fat-soluble vitamins were given by dropper to provide each rat 162 IU of vitamin A and 1.62 IU of vitamin D per week. *dl*-α-Tocopherol was given weekly to provide 10 mg/rat.

Total selenium was determined by wet-ashing the samples, isolation of selenium by coprecipitation with arsenic and measuring the 4,5-benzopiazselenol fluorometrically.[12]

EXPERIMENTAL

Experiment 1. Figure 1 shows typical retention curves when 1 or 260 μg of selenium (0.6–0.8 μCi) as sodium selenite were injected into 130-g rats fed the casein basal diet. With the larger dose there was a characteristic initial rapid loss of ^{75}Se, a transitional period of about 14 days, followed by a stable slow rate of release which continued for the remainder of the 6-month test period. With the smaller dose of selenite, retention or elimination was also characterized by 3 phases, but with a much larger fraction of the dose still present at the beginning of the stable phase. Nevertheless, the absolute amounts of selenium in the 2 groups were still markedly different at the beginning of the stable phase, 50 μg for the 260-μg dose, and 0.75 μg when 1 μg had been injected. Supplementation of 2 groups of rats with 83 mg of *dl*-α-tocopherol per rat 3 times weekly failed to alter the rate of elimination of either dose of selenium.

Experiments 2 and 2a. The percentage retentions for 5 doses of selenium are presented in figure 2. In this experiment 25 male weanling Holtzman rats were fed the basal diet for 2 weeks prior to injection (exp. 2), and 25 others were fed the basal diet for 103 days before injection (exp. 2a). The first groups averaged 114 to 122 g at the time of injection; the second, 384 to 394 g. In each series, groups of 5 rats were injected with 1 to 260 μg of selenium as selenite (0.4–0.7 μCi), and the radioactivity remaining in the rats was measured at intervals for

[9] Distillation Products Industries, Rochester, New York.
[10] The mineral premix used in Torula diets contained the following: (in per cent) $CaCO_3$, 59.35; NaCl, 27.94; KCl, 11.17; MgO, 0.11; $FePO_4$, 1.23; KIO_3, 0.12; $MnSO_4 \cdot H_2O$, 0.07; and $CoCl_2 \cdot 6H_2O$, 0.027.
[11] See footnote 7.
[12] See footnote 2.

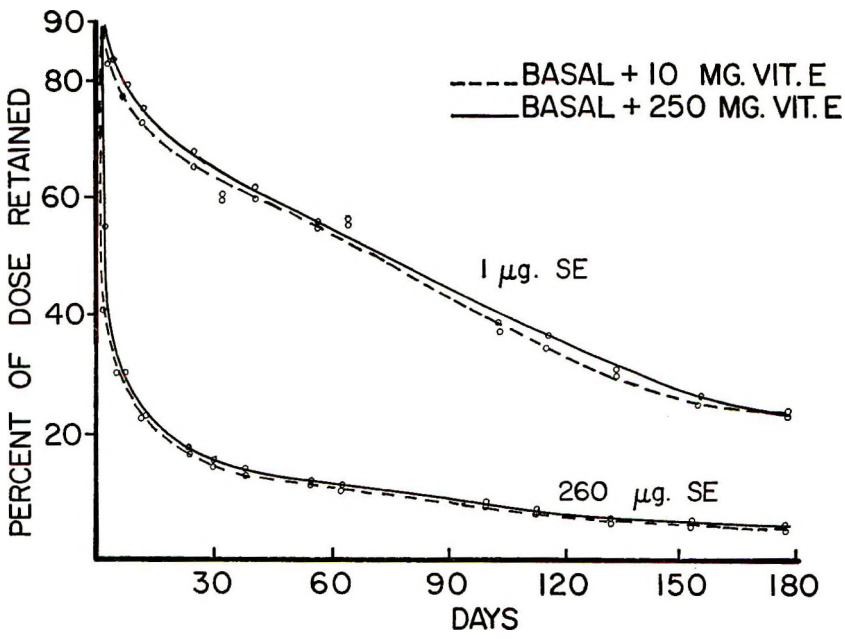

Fig. 1 Retention of 2 levels of ^{75}Se-labeled selenite injected into rats fed the basal diet with or without vitamin E supplementation. Data are plotted on a linear scale (exp. 1).

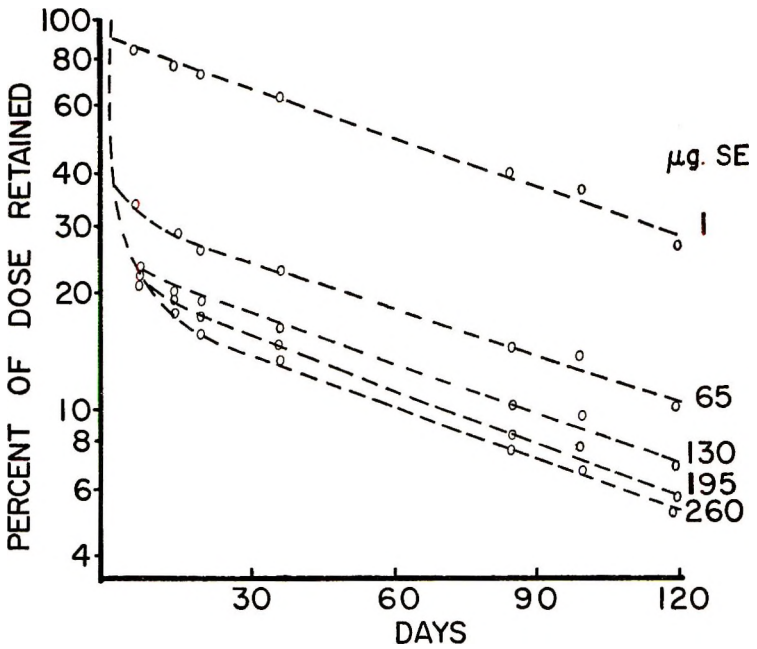

Fig. 2 Retention of different doses of ^{75}Se-labeled selenite by rats fed the basal diet 14 days prior to and after injection. Data are plotted on a semi-logarithmic scale (exp. 2).

118 days for the smaller animals and for 91 days for the larger ones.

The latter phases of all retention curves for ^{75}Se were linear on the semi-log scale, and all lines were parallel. Thus, first-order reaction kinetics could be applied to the data, the rate of loss of fixed selenium being similar at all levels of injected selenium. When heavier, older animals were used, similar results were observed.

The major differences between rats injected with different levels of selenium were in the percentages of the doses estimated to be in the fixed pool shortly after injection, and in the amounts of fixed ^{75}Se present at all times (table 1). When higher levels were injected, the percentages retained initially were much less than when small doses were given, although the absolute amounts of injected selenium in the fixed pool were much greater with the higher dosages. But despite a 50-fold difference in absolute amount, values for the biological half-life of the fixed ^{75}Se were remarkably similar for all dose levels: an average of 70 days for the lighter rats and 72.7 for the heavier rats. Body size, therefore, apparently played no significant role in the unloading of fixed selenium.

Experiment 3. In an attempt to alter the retention of fixed selenium, male weanling Holtzman rats were fed the basal diet for 2 weeks, injected subcutaneously with 260 μg of selenium as selenite containing ^{75}Se, and allowed to lose selenium for 14 days. The radioactivity retained by the animals was then determined; the rats were divided into treatment groups of similar initial stable retention, and fed one of the following diets: the basal diet; a fortified basal diet which contained 36% casein, 0.5% methionine, and 20 times as much of the water-soluble vitamins as in the basal diet (7); a diet in which 20% of linseed meal replaced 10% of the casein and 10% of the sucrose in the basal diet; the basal diet with 0.5 ppm of added selenium as selenite; or laboratory ration. Each of these diets has been shown previously to alter selenium metabolism under other circumstances (6–8, 11). Figure 3 shows the effects of these diets on the retention of selenium when expressed as a percentage of the original dose. The rate of loss of the labeled selen-

TABLE 1

Retention of different amounts of injected selenium by large and small rats

Selenium injected	Maximal retention [1]		Biological half-life [2]
μg	%	*μg*	*days*
Small rats, experiment 2 [3]			
1	86.1	0.9	71.7 ± 3.5 [a,4]
65	31.2	20.3	74.7 ± 1.4 [a]
130	22.7	29.5	71.2 ± 1.5 [a]
195	21.2	41.3	65.1 ± 2.2 [a]
260	19.5	50.4	63.6 ± 0.7 [a]
Large rats, experiment 2a [5]			
1	82.1	0.8	71.4 ± 1.4 [a,b]
65	43.6	28.3	67.8 ± 5.4 [b]
130	26.1	33.9	78.9 ± 1.1 [a]
195	18.4	35.9	74.0 ± 2.1 [a,b]
260	16.2	42.1	70.2 ± 2.1 [a,b]

[1] Maximal retention was calculated by extrapolating the linear third phase of retention curve to the injection time.

[2] The biological half-life was determined by calculating the regression coefficient between the logarithm of retention and time and converting to a first order rate constant (and to a biological half-life) for each rat. Biological half-life equalled

$$\frac{(n\Sigma X^2 - (\Sigma X)^2)\,(0.6931)}{(n\Sigma XY - \Sigma X \Sigma Y)\,(-2.302)}$$

where X is the time after injection, Y the logarithm of the per cent of the dose retained, n the number of time intervals after injection, and Σ the summation of values for a particular rat. The biological half-lives reported are the averages for 5 rats plus or minus the standard error except when 260 *μg* of selenium was injected (2–3 rats).

[3] Weanling rats were fed the basal diet for 14 days and then were injected with the indicated quantity of radioactive selenium as selenite. The rats weighed 114 to 122 g when injected.

[4] Any two means with the same superscript within weight groups are not significantly different ($P < 0.05$) by Duncan's multiple range test (15).

[5] Weanling rats were fed the basal diet for 103 days prior to injection with the indicated quantity of radioactive selenium as selenite. The rats weighed 384 to 394 g when injected.

ium was least from the rats fed the basal diet and greatest from those receiving 0.5 ppm of selenium during the depletion period, either as inorganic selenite added to the basal diet or as selenium provided in the natural ingredients of the laboratory ration. Intermediate rates of loss of labeled selenium resulted when the other diets were fed. Most of the retention curves deviated from the straight lines seen in the other experiments, presumably reflecting adjustments to the diets instituted after the elimination of fixed selenium had begun.

The rates of elimination varied with the selenium content of the diets fed. The basal diet, which showed the slowest rate of selenium unloading, had the lowest selenium content, 0.04 ppm. There was a slightly higher rate of excretion when the fortified casein-methionine diet was fed (estimated 0.09 ppm of selenium). Linseed meal contained 1.3 ppm of selenium; the diet fed, an estimated 0.28 ppm and with this diet an intermediate rate of loss occurred. The laboratory ration with which loss was relatively rapid, contained 0.5 ppm of selenium (8).

Experiment 4. Effects of graded levels of dietary selenium upon the retention of labeled selenium in the fixed pool are presented in table 2. In this experiment weanling rats were fed the basal diet for 2 weeks prior to and for 7 days after injection with 10 μg of selenium as selenite (0.3 μCi of ^{75}Se). The rats were then divided into 8 groups of 5 rats each with similar retentions and fed the following diets: the basal diet plus graded levels of selenium as selenite, the laboratory ration, the Torula diet or the Torula diet supplemented with 0.05 ppm of selenium as selenite. The experiment was continued until the radioactivity of the rat had decreased to less than twice the background of the Armac counter.

The rats fed the basal diet or the Torula diets lost selenium at a constant rate over a 7-month depletion period. Those receiving selenium in the basal diet after the labeled dose had been given showed a period of adjustment during which the initial rate of selenium loss was more

TABLE 2

Effect of diet on the biological half-life of selenium (exp. 4)

Diet	Biological half-life [1]	
	Phase 1 [2]	Phase 2
	days	*days*
Torula II	—	61.9 ± 3.2 [c,3]
Torula II + 0.05 ppm Se	—	77.5 ± 2.0 [a]
Basal	—	70.2 ± 1.6 [b]
Basal + 0.05 ppm Se	44.4 ± 0.9 [a]	56.9 ± 0.8 [d]
Basal + 0.15 ppm Se	23.1 ± 0.5 [b]	43.0 ± 1.0 [e]
Basal + 0.45 ppm Se	16.2 ± 0.3 [c]	31.6 ± 0.4 [f]
Basal + 0.95 ppm Se	13.5 ± 0.1 [c]	27.2 ± 0.2 [g]
Laboratory ration	15.9 ± 0.1 [c]	33.7 ± 0.2 [f]

[1] See footnote to table 1.

[2] Phase 1 gives the biological half-life of the injected selenium during the adjustment to the diets containing higher levels of selenium. Phase 2 is the biological half-life of the injected selenium during the stable phase of the depletion period.

[3] Average of 5 rats ± SE. Any 2 means within a phase that have the same superscript are not significantly different ($P < 0.05$) by Duncan's multiple range test (15).

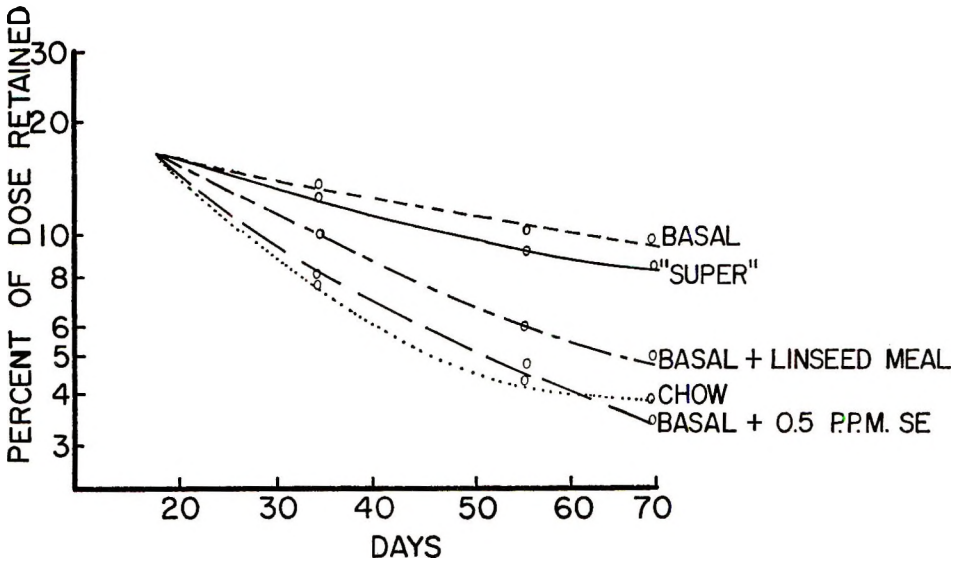

Fig. 3 Effect of diet on the loss of fixed radioactive selenium. Weanling rats were fed the basal diet 14 days and then injected with 260 μg of radioactive selenite. The rats were distributed to groups with equal retentions 14 days after injection and fed the test diets (exp. 3).

rapid than during the latter phase of the curve. The biological half-life of the injected selenium, for both phases, revealed significant decreases for each increment of unlabeled selenium in the diet (table 2).

The retention of selenium by rats fed the Torula diet, which represented a possible selenium deficiency (0.005 ppm), was similar to the retention by rats fed the basal diet. The biological half-life of the fixed pool of selenium was actually less with the Torula diet than with the basal diet, although the reverse might have been expected. The rate of depletion of selenium from rats fed the basal diet plus 0.45 ppm of Se and the calculated half-life were also very similar, 15.9 vs. 16.2 days during the transition phase, and 33.7 vs. 31.6 days after equilibrium was established (table 2).

The marked dependence of retention upon the selenium content of the diet during the depletion period (table 2) together with the fact that the rates of unloading with diets such as the laboratory ration, linseed meal, and high protein-methionine (fig. 3) varied with the selenium content of these diets, suggested that the ingredients themselves did not affect the elimination of fixed selenium except as they involved changes in selenium consumption. Since these ingredients were selected because of their influence on volatilization (6–8), or on the severity of chronic selenium toxicity (11), their lack of effect on fixed selenium supports the conclusion that a factor capable of modifying one aspect of selenium metabolism frequently is inactive under other circumstances.

Experiment 5. Figure 4 shows the effect of 1.0 ppm of selenium as selenite in the basal diet on the retention of 1 μg of injected selenium (0.1 μCi ^{75}Se) by rats. The presence of selenite in the diet greatly increased the amount of selenium lost initially and also increased the subsequent rate of excretion. Total selenium was determined chemically at intervals during the experiment and table 3 shows that the selenium content of liver and blood from animals receiving supplemental dietary selenium was approximately 3 times that of rats fed the basal diet. With either diet the levels of total selenium in the blood and liver remained relatively constant throughout the experimental period.

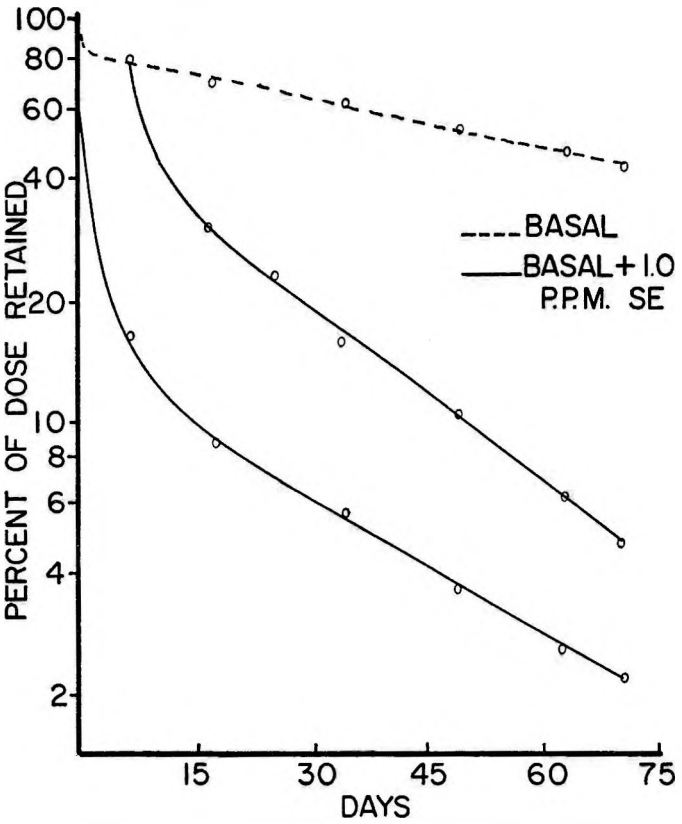

Fig. 4 Effect of dietary selenite on retention of injected ^{75}Se-labeled selenite. Weanling rats were fed the basal diet or the basal + 1 ppm selenium 14 days prior to and after injection with 1 μg of selenium as selenite. Groups of rats were transferred from the basal diet to the supplemented diet 7 days after injection (exp. 5).

TABLE 3

Effect of dietary selenium on the selenium content of rat tissues

Supplemental selenium	Days [1]				
	— 14	0	7	35	70
	Liver, ppm dry matter				
None	3.31 ± 0.03 [2]	0.91 ± 0.03	0.93 ± 0.06	1.01 ± 0.03	1.75 ± 0.10
1 ppm Se	3.13 ± 0.01	4.42 ± 0.47	3.75 ± 0.10	3.63 ± 0.33	3.91 ± 0.01
1 ppm Se [3]	—	—	—	3.58 ± 0.09	4.05 ± 0.02
	Blood, μg/ml				
None	0.39 ± 0.02	0.25 ± 0.02	0.22 ± 0.01	0.22 ± 0.01	0.35 ± 0.01
1 ppm Se	0.40 ± 0.02	0.45 ± 0.06	0.42 ± 0.01	0.64 ± 0.01	0.58 ± 0.02
1 ppm Se [3]	—	—	—	0.52 ± 0.01	0.61 ± 0.01

[1] The groups of rats were fed the basal diet with or without supplementation and injected with 1 μg of radioactive selenium as selenite at zero days.
[2] Average of 5 rats ± SE.
[3] Groups of rats were transferred from the basal diet to the basal supplemented with 1.0 ppm of selenium as selenite 7 days after injection with radioactive selenite.

When rats were transferred to the selenium-supplemented diet 7 days after selenite injection, a period of rapid loss was observed followed by a period when the rate of excretion was similar to that of rats fed the supplemented diet continuously (fig. 4). The total selenium content of blood and liver of rats transferred to the supplemented diet was similar to the content of rats that received the diet from weaning (table 3).

DISCUSSION

That there are two distinct phases in the rate of elimination of selenium has been observed in sheep (12) and rats (13) and in the tissues of rats (1,2), chickens (4), dogs (3,9) and sheep (12, 14). Blincoe (13) considered the depletion curve of selenite injected into rats to represent two first-order reactions and calculated rate constants and biological half-life for the initial and final phases. His rats were fed a stock diet of unspecified selenium content, they were observed for only 14 days after the selenite was injected, and the calculated value for the biological half-life during the slower phase was found to be 14 days; in the present study the half-life for rats fed the laboratory ration was 15.9 days during a comparable time period (table 2, phase 1), but over longer time intervals and especially with other diets the half-life of the fixed selenium was much longer, usually about 70 days (table 1; table 2, basal).

Many workers have demonstrated that injected selenite is rapidly bound to protein (2–5) and that over a few days the percentage of the remaining selenium that is bound or fixed to protein approaches 95% (2,12). The amount of "fixed selenium" retained by our rats increased as the amount of injected selenite was increased, but when expressed as a percentage of the dose, the percentage retained decreased with increasing dose levels. Selenium-75 in the fixed pool left the body at a constant rate, which depended upon the level of selenium in the diet. Other factors such as increased protein and methionine, or the laboratory diet, which enhance the volatilization of subacute injections of selenium (6–8), or linseed meal, which alleviates chronic selenium toxicity (11), exerted no significant effect on the excretion of fixed selenium except as these ingredients varied in selenium content.

The addition of selenite to the diet after a constant rate of excretion had been established resulted in a rapid increase in the rate of loss of ^{75}Se (fig. 4), and an increase in total selenium in blood and liver (table 3). A stable rate of loss and constant total selenium levels were observed during the final phase of the experiment. Since the total selenium levels of blood and liver were constant even when the daily intake of selenium was high, the rat appeared to be in selenium balance and was excreting as much selenium as was consumed. If 2 pools of selenium are postulated, the fixed pool appears to increase at a decreasing rate since the percentage of the dose retained by the rat decreased with increasing dose levels (table 2). This suggests that the free pool must increase with increasing dose levels and since the two are in equilibrium when the dietary level of selenium is increased, the free pool would increase and any fixed ^{75}Se entering the large free pool of stable selenium would probably be excreted.

ACKNOWLDEGMENT

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Vitamin B_6 Deficiency in Rats: Utilization of ^{14}C-labeled glutamic acid and sodium 2-ketoglutarate [1]

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ABSTRACT The effect of a vitamin B_6 deficiency on the utilization of ^{14}C-labeled glutamic acid and 2-ketoglutarate in brain and liver tissue was investigated. When glutamate-2-^{14}C was injected the activity in the expired carbon dioxide from vitamin B_6-depleted rats was less than that from the control rats. Three fractions were isolated from the brain and liver hydrolysates: 1) all amino acids minus glutamic and aspartic acid, 2) glutamic acid, and 3) aspartic acid. There was no difference in the proportion of ^{14}C found in the 3 fractions isolated from the brains of the deficient group as compared with the control group except in the ^{14}C activity of aspartic acid. In this instance the least ^{14}C activity was noted in the pyridoxine-supplemented group. Liver amino acid fractions from the vitamin B_6-deficient rats showed lower levels of ^{14}C activity. Variable levels of $^{14}CO_2$ were obtained upon injection of 2-ketoglutarate-3,5-^{14}C. In the brain fractions 1 and 3 were significantly lower in the vitamin B_6-deprived group than in the vitamin-supplemented group. The 3 fractions from vitamin B_6-deficient livers showed lower levels of ^{14}C activity than from the control rats, but the difference was only significant with fraction 3.

Vitamin B_6 functions as a coenzyme for many metabolic reactions. One of the most important of these is transamination. Previous investigators have measured the activity of the transaminase enzymes in order to ascertain the effect of various factors on transamination (1–13). However, radioactive compounds provide another avenue of approach to this problem. Tracer techniques could be used to note the rate of formation of an amino acid from the corresponding keto acid, such as the formation of glutamic acid from 2-ketoglutarate and in the rate of formation of the keto acid from the corresponding amino acid. If this method were successful than it could be used to study the effect of various factors on transaminase activity. Therefore, in the present study we have investigated the effect of a pyridoxine deficiency on the utilization of ^{14}C-labeled glutamic acid and 2-ketoglutarate in brain and liver.

MATERIALS AND METHODS

Male weanling rats weighing between 40 to 60 g of the CFN strain [3] were used. They were fed the following 3 diets ad libitum for 6 weeks; diet 1, vitamin B_6-deficient [4] consisting of the following: (in per cent) vitamin-test casein, 18; sucrose, 68; vegetable oil, 10; USP salt mixture no. 2, 4. The vitamin mixture contained: (in g/45.5 kg diet) vitamin A conc (200,000 units/g), 4.5; vitamin D conc (400,000 units/g), 0.25; α-tocopherol, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; *p*-aminobenzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; thiamine·HCl, 1.0; Ca pantothenate, 3.0; biotin, 0.020; folic acid, 0.090; and vitamin B_{12}, 0.00135. Diet 2 was the vitamin B_6-deficient diet plus 50 μg of pyridoxine·HCl [5] per rat per day, and diet 3 consisted of dog food [6] with the following composition: crude protein not less than 24.0%, crude fat not less than 7.0% and crude fiber not more than 3.0%. The ingredient formula consisted of toasted yellow corn flakes, animal fat, meat meal, dehulled solvent-extracted soybean meal, vitamin A palmitate, vitamin D-activated animal sterol, calcium pantothenate, niacin, riboflavin, vitamin B_{12}, vitamin E, vitamin K, dried brewer's yeast, minerals, potassium iodide and salt 0.75%.

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[5] See footnote 4.

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TABLE 1

Rat, brain and liver weights (rats injected with glutamic acid-2-^{14}C)

Diet	Wt of rat	Wt of brain	Wt of liver
	g	*g*	*g*
Dog food [1]	265 ± 7 [2]	1.62 ± 0.06	11.1 ± 0.33
Vitamin B_6-deficient diet + vitamin B_6	227 ± 9	1.38 ± 0.07	10.9 ± 0.54
Vitamin B_6-deficient	98 ± 5	1.60 ± 0.02	4.2 ± 0.25

[1] See text for ingredient formula.
[2] Mean of 8 rats ± SE of mean.

TABLE 2

Rat, brain and liver weights (rats injected with 2-ketoglutarate-3,5-^{14}C)

Diet	Wt of rat	Wt of brain	Wt of liver
	g	*g*	*g*
Dog food [1]	263 ± 38 [2]	1.85 ± 0.05	10.7 ± 0.64
Vitamin B_6-deficient diet + vitamin B_6	221 ± 25 [2]	1.75 ± 0.04	11.1 ± 0.98
Vitamin B_6-deficient	95 ± 2 [3]	1.62 ± 0.04	4.4 ± 0.32

[1] See text for ingredient formula.
[2] Mean of 5 rats ± SE of mean.
[3] Mean of 6 rats ± SE of mean.

After 6 weeks each rat was injected intravenously with 10 μCi of either glutamic acid-2-^{14}C (specific activity, 1.44 mCi/mmole) or sodium 2-ketoglutarate-3,5-^{14}C (specific activity, 6.9 mCi/mmole).[7] The animals were then placed in a closed glass metabolism cage and the respired carbon dioxide was collected in sodium hydroxide. At the end of one hour the rats were bled by means of cardiac puncture. Livers and brains were removed for analysis.

The sodium hydroxide solutions containing the respired carbon dioxide were made up to a volume of 500 ml. Four-tenths milliliter was plated onto a stainless steal planchet, the water evaporated and the residue counted.

An aliquot of blood (0.2 ml) was plated directly onto planchets, dried and counted.

Each tissue was refluxed with 6 N hydrochloric acid for 24 hours and then evaporated to dryness several times to remove the excess hydrochloric acid. The dried hydrolysate was made up to a volume of 10 ml with 10% isopropyl alcohol. Approximately 70 mg of the hydrolysate were used for the isolation of amino acids. The procedure of Hirs et al. (14) was followed. Three fractions were eluted from the Dowex 1 X-8 column with 0.5 N acetic acid: 1) fraction A, all amino acids except glutamic and aspartic acid; 2) glutamic acid ; and 3) aspartic acid. A buffered ninhydrin reagent (15) was used to locate these amino acid fractions. The fractions were combined, evaporated to dryness and then plated as the hydrochloride salts for counting. Paper chromatograms were run on the glutamic and aspartic acid fractions to determine their purity.

The system used for radioactivity counting consisted of an FD-1 flow counter, a P-31 Geiger preamplifier, an SC 100 Multi/matic sample changer, an SC-71 Compu/matic scaler and an SC-87A Auto/printer from Tracerlab.[8] Matheson gas (98.7% helium and 1.3% butane) was passed through the flow counter. Corrections were made for self-absorption.

RESULTS

After the rats had been fed the vitamin B_6-deficient diet for 3 weeks, their body weights remained at slightly less than 100 g. The first signs of acrodynia were evident at 4 weeks. The weights of the rats, their livers and brains at the time of killing are shown in tables 1 and 2.

Pyridoxine-deficient rats showed low levels of ^{14}C activity in carbon dioxide and blood when injected with glutamic acid-2-^{14}C (table 3). The ^{14}C activity in the amino acid fractions isolated from the brain and liver hydrolysates of these rats is shown in tables 4 and 5. In the brain the ^{14}C activity of the glutamic acid and amino acid fractions did not vary significantly in the three dietary groups. The

[7] Tracerlab, Waltham, Massachusetts.
[8] See footnote 7.

^{14}C level of the aspartic acid fraction of the pyridoxine-deficient group was significantly higher than that of the pyridoxine-supplemented group but lower than the value for the group fed dog food. In the liver the ^{14}C activity level was lower ($P < 0.01$) in the vitamin B_6-deficient group for all 3 fractions.

The carbon dioxide ^{14}C activity from rats injected with 2-ketoglutarate-3,5-^{14}C was extremely variable for the three dietary groups (table 6). Lower levels of ^{14}C activity were noted in the blood from the depleted rats (table 6). Tables 7 and 8 show the ^{14}C activity found in the brain and liver hydrolysates. Lower amounts of ^{14}C were found in the amino acid fractions from the depleted rats in both the brain and liver hydrolysates. The activity of ^{14}C in the glutamic acid isolated from the vitamin B_6-depleted rats was lower but not significantly, both in the liver and brain. Aspartic acid ^{14}C activity was lower in both tissues from the deficient rats. However, in the liver it was significantly lower ($P < 0.01$), whereas in the brain it was signifi-

TABLE 3

The ^{14}C activity [1] in carbon dioxide and blood one hour after injection of 10 μCi of glutamic acid-2-^{14}C

Diet	$^{14}CO_2$	Blood
	count/min [2]	*count/min* [3]
Dog food [4]	338 ± 59 [5] **	258 ± 15 **
Vitamin B_6-deficient diet + vitamin B_6	322 ± 218	256 ± 8 **
Vitamin B_6-deficient	84 ± 15	192 ± 9

[1] Adjusted for equal dilution of tracer in 100-g rat.
[2] Counts/minute = $\frac{\text{body weight}}{100}$ × counts/minute of $\frac{1}{1250}$ $\frac{(0.4\text{ ml})}{(500\text{ ml})}$ CO_2 expired in one hour.
[3] Counts/minute = $\frac{\text{body weight}}{100}$ × counts/minutes of 0.2 ml blood.
[4] See text for ingredient formula.
[5] Mean of 8 rats and SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 4

The ^{14}C activity of amino acid fractions of brain one hour after injection of 10 μCi of glutamic acid-2-^{14}C

Diet	Glutamic acid	Aspartic acid	Amino acids
	count/min [1]	*count/min* [1]	*count/min* [1]
Dog food [2]	2570 ± 326 [3]	500 ± 65	3234 ± 327
Vitamin B_6-deficient diet + vitamin B_6	2311 ± 251	314 ± 30 *	2937 ± 182
Vitamin B_6	2289 ± 144	438 ± 30	2888 ± 339

[1] Counts/minute per total brain per 100-g rat.
[2] See text for ingredient formula.
[3] Mean of 8 rats ± SE of mean.
* Significantly different from the deficient mean, $P < 0.05$.

TABLE 5

The ^{14}C activity of amino acid fractions of liver one hour after injection of 10 μCi of glutamic acid-2-^{14}C

Diet	Glutamic acid	Aspartic acid	Amino acids
	count/min [1]	*count/min* [1]	*count/min* [1]
Dog food [2]	237,000 ± 31,000 [3] **	23,500 ± 2,400 **	78,200 ± 10,500 **
Vitamin B_6-deficient diet + vitamin B_6	209,000 ± 31,000 **	21,000 ± 3,600 **	90,600 ± 10,400 **
Vitamin B_6-deficient	73,000 ± 6,000	7,600 ± 500	30,200 ± 2,100

[1] Counts/minute per total liver per 100-g rat.
[2] See text for ingredient formula.
[3] Mean of 8 rats ± SE of mean.
** Significantly different from the deficient mean, $P < 0.01$.

TABLE 6

The ^{14}C activity [1] in carbon dioxide and blood one hour after the injection of 10 μCi of 2-ketoglutarate-3,5-^{14}C

Diet	No. of rats	$^{14}CO_2$	Blood
		count/min [2]	*count/min* [3]
Dog food [4]	5	967 ± 321 [5]	307 ± 123
Vitamin B_6-deficient diet + vitamin B_6	5	577 ± 480	366 ± 15 **
Vitamin B_6-deficient	6	557 ± 71	194 ± 12

[1] Adjusted for equal dilution of tracer in 100-g rat.

[2] Counts/minute = $\frac{\text{body weight}}{100}$ × counts/minute of $\frac{1}{1250}$ $\frac{(0.4\text{ ml})}{(500\text{ ml})}$ CO_2 expired in one hour.

[3] Counts/minute = $\frac{\text{body weight}}{100}$ × counts/minute of 0.2 ml blood.

[4] See text for ingredient formula.

[5] SE of mean is included.

** Significantly different from the deficient mean, $P < 0.01$.

TABLE 7

The ^{14}C activity of amino acid fractions of brain one hour after injection of 10 μCi of 2-ketoglutarate-3,5-^{14}C

Diet	No. of rats	Glutamic acid	Aspartic acid	Amino acids
		count/min [1]	*count/min* [1]	*count/min* [1]
Dog food [2]	5	5,612 ± 2,310 [3]	1,004 ± 191	7,115 ± 196 **
Vitamin B_6-deficient diet + vitamin B_6	5	5,420 ± 961	962 ± 52 *	6,872 ± 470 *
Vitamin B_6-deficient	6	4,276 ± 494	705 ± 85	5,189 ± 527

[1] Counts/minute per total brain per 100-g rat.

[2] See text for ingredient formula.

[3] SE of mean is included.

** Significantly different from the deficient mean, $P < 0.01$.

* Significantly different from the deficient mean, $P < 0.05$.

TABLE 8

The ^{14}C activity of amino acid fractions of liver one hour after injection of 10 μCi of 2-ketoglutarate-3,5-^{14}C

Diet	No. of rats	Glutamic acid	Aspartic acid	Amino acids
		count/min [1]	*count/min* [1]	*count/min* [1]
Dog food [2]	5	303,000 ± 139,000 [3]	60,600 ± 6.500 **	208,000 ± 22,000
Vitamin B_6-deficient diet + vitamin B_6	5	331,000 ± 122,000	52,800 ± 6,600 **	211,000 ± 113,000
Vitamin B_6-deficient	6	95,000 ± 16,000	14,400 ± 1,100	60,000 ± 19,000

[1] Counts/minute per total liver per 100-g rat.

[2] See text for ingredient formula.

[3] SE of the means is included.

** Significantly different from the deficient mean, $P < 0.01$.

cantly lower ($P < 0.05$) only when compared with the pyridoxine-supplemented group.

DISCUSSION

When pyridoxine-deficient rats were injected with radioactive glutamic acid, the ^{14}C activity in the expired air was less than that of the controls. This indicates that the metabolism of glutamic acid for energy purposes is at a lower rate in the vitamin B_6-deficient rats. Since a number of reactions such as transamination, deamination and decarboxylation require pyridoxine, this is not unexpected. However, when tracer ketoglutarate was used, the depleted rats appeared to oxidize the tracer as well as the rats receiving pyridoxine. This may be attributed to extremely variable results. Variable values were also obtained when the respired carbon dioxide was monitored in a flow chamber of a vibrating reed electrometer.[9]

[9] Thiele, V. F., and Myron Brin. The metabolism of ^{14}C-labeled amino and keto acids in normal and vitamin B_6-deficient rats. Abstracts 150th Meeting of the American Chemical Society, Atlantic City, 1965.

Vitamin B_6-deficient rats when injected with the tracer glutamic acid showed the same amount of radioactivity in the glutamic acid, aspartic acid and remaining amino acid fractions isolated from the brain as did the control rats, except for the aspartic acid ^{14}C fraction which was lower in the pyridoxine-supplemented group. The ratio of the ^{14}C activity of the aspartic acid fraction and the ^{14}C activity of the remaining amino acid fraction off the column to the ^{14}C activity of the glutamic acid is the same in each case, 1.00:0.19:1.26 for dog food, 1.00:0.14:1.27 for vitamin B_6-deficient + vitamin B_6, and 1.00:0.15:1.27 for the vitamin B_6-deficient group. Therefore, the extent of conversion of glutamic acid to the other amino acids must be the same in each case in the brain. This was not expected since pyridoxine functions as a coenzyme for the transamination reaction involving glutamic acid. Also Ames and his associates (2) reported a decrease in transaminase activity in the brains of pyridoxine-deficient rats.

When the tracer 2-ketoglutarate was used the amino acid fractions of the brain hydrolysates from the pyridoxine-deficient rats showed lower activity than the controls. This difference was highly significant in the aspartic acid and amino acid fractions when compared with the supplemented group. The ratio of ^{14}C activity of the aspartic acid and the ^{14}C activity of the amino acid fractions to the ^{14}C activity of the glutamic acid was the same as those obtained with the tracer glutamic acid, 1.00:0.18:1.27 for dog food, 1.00:0.18:1.27 for vitamin B_6-deficient + vitamin B_6, and 1.00:0.16:1.21 for vitamin B_6-deficient rats. Despite the vitamin B_6 deficiency, the brain converted glutamic acid to aspartic acid and to other amino acids. The 2-ketoglutarate was also converted to glutamic acid, aspartic acid, and other amino acids in the brains of the depleted rats.

A decrease in transaminase activity in pyridoxine-deficient rats has been reported by many investigators (1–3, 6, 9, 10). The data obtained both with glutamic acid and 2-ketoglutarate showed low levels of ^{14}C activity in the amino acid fractions isolated from the livers of depleted rats. This indicated that a lack of pyridoxine caused a decrease in transaminase activity in the liver, since the vitamin functions as a coenzyme in the transamination reaction. While the lowest level of ^{14}C occurs in the vitamin B_6-deficient group for all 3 fractions, the ratio of the ^{14}C activity of the aspartic acid fraction and the ^{14}C activity of the remaining amino acid fractions off the column to the ^{14}C activity of the glutamic acid is the same in each case when the tracer glutamic acid was used, 1.00:0.10:0.33 for dog food, 1.00:0.10:0.43 for vitamin B_6-deficient +vitamin B_6, and 1.00:0.10:0.41 for vitamin B_6-deficient rats. The corresponding ratios after the injection of 2-ketoglutarate are 1.00:0.20:0.69; 1.00:0.16:0.64 and 1.00:0.15:0.63.

When these ratios are compared with those for the brain, the degree of conversion of glutamic acid to aspartic acid is similar for both organs with the tracer 2-ketoglutarate. This is not the case in the conversion of glutamic acid to the remaining amino acids. Many different types of reactions take place in the liver and thus it is probable that these amino acids are rapidly shunted off into other metabolic pathways.

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Effects of Excess Leucine on Growth and Food Selection [1]

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ABSTRACT When 5% leucine was added to a 9% casein diet supplemented with methionine, growth and food intake of rats were depressed. Although isoleucine and valine were found to largely overcome this growth depression the initial reduction in weight and complete restoration to the control rate of growth resulted only after additional supplementation with tryptophan, phenylalanine and threonine. When rats were given a choice between diets containing 5% leucine and a protein-free diet, they invariably chose the protein-free diet. About half of the rats chose the protein-free diet even when the high leucine diet was "corrected" by supplementation with isoleucine, valine, tryptophan, phenylalanine, and threonine. Supplementation of the high leucine diet with a complete amino acid mixture devoid of leucine increased selection of the high leucine diet. Thus, it appears that rats will avoid almost all diets containing 5% leucine, even if the alternate is a much inferior, protein-free diet.

The growth rate of young rats fed a low protein diet containing excess leucine is much less than that of control animals fed the low protein diet alone. This effect has been attributed to a leucine-isoleucine and valine antagonism because supplementation of the high leucine diet with small amounts of isoleucine and valine, which are not the growth-limiting amino acids in the control diet, will alleviate the growth depression (1). The effectiveness of isoleucine and valine in preventing the growth depression depends upon the amount of leucine included in the diet. Even high levels of isoleucine and valine do not restore to normal the growth of rats fed a diet containing 9% of casein and 5% of leucine (2), yet this amount of leucine does not markedly depress the growth rate of rats fed a diet containing 18% of casein. The growth-depressing effect of a high leucine intake therefore appears to be the result of a more complex amino acid interrelationship than the leucine-isoleucine and valine antagonism originally postulated.

The results presented below indicate that amino acids other than isoleucine and valine are required to prevent retardation of the growth of rats fed a 9% casein diet in which 5% of L-leucine has been included. Some observations on the effect of excess leucine on the growth and food intake of rats fed for only 2 hours daily are also described.

Investigation of food preferences of rats offered a choice between a protein-free diet and various diets in which amino acid imbalances had been created showed that the rat selected a protein-free diet in preference to diets that would support growth but in which the amino acid pattern was unbalanced. The rat also showed a clear preference for a diet with a balanced amino acid pattern rather than one in which the amino acid pattern was not in balance (3). Food preferences of rats offered a choice between a protein-free diet and various low protein diets containing excess leucine were therefore investigated and the results of some of these studies are also presented.

EXPERIMENTAL PROCEDURE

Male albino rats were used throughout these experiments. All rats were housed in individual suspended cages with screen

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TABLE 1

Effect of methionine and threonine on leucine-isoleucine and valine antagonism

Diet				Wt gain/2 weeks		
Casein	L-Leu	DL-Ileu	DL-Val	No additions	+ 0.3% DL-Met	+ 0.3% DL-Met + 0.36% DL-Thr
	%	%	%	g	g	g
9	—	—	—	29 ± 1 [1]	37 ± 3	50 ± 4
9	5	—	—	22 ± 4	12 ± 2	11 ± 3
9	5	0.3	0.3	29 ± 3	22 ± 3	27 ± 3

[1] SE of mean.

bottoms and were weighed at regular intervals during the experimental periods.

The diets used contained 4% (4) or 5% (5) of salts, 5% of corn oil, 1% (6)[5] or 0.5% (5)[6] of vitamins and 0.2% choline chloride. Sucrose was included to make each diet up to 100%. Casein and amino acids were added to the diets at the expense of sucrose. Water was supplied to the rats ad libitum, and the diets were stored under refrigeration.

In the experiments on the effects of amino acid supplementation three control diets were used; namely, a basal diet containing 9% casein; a high leucine diet containing 9% casein and 0.74% glutamic acid plus 5% L-leucine to produce leucine-isoleucine antagonism; and a high leucine diet containing 9% casein and 5% L-leucine but supplemented with 0.32% DL-isoleucine and 0.3% DL-valine to alleviate the antagonism. Glutamic acid was added to make the two high leucine diets used in the growth studies isonitrogenous.

For the studies on the physical form of the diet, three experimental diets mentioned above were supplemented with 0.3% DL-methionine and made into a gel in the following manner. All of the dry components of the diet except choline chloride and the vitamins were weighed and mixed together. A measured amount of agar-agar equivalent to 4% and an amount of water equal to the total weight of the dry components were mixed and brought to a boil. When the agar-agar was completely dissolved, the solution was immediately added to the dry components; and the resulting suspension was mixed vigorously. Then the vitamins and choline chloride were added, and the complete diet was again mixed. When the diet began to thicken and became homogeneous, it was

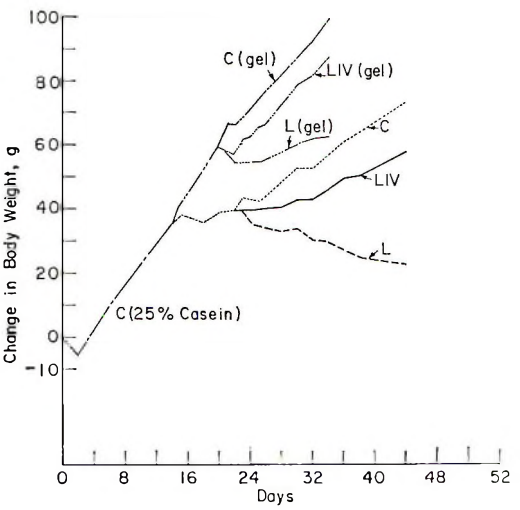

Fig. 1 All rats were fed the 25% casein (dry) diet (— — —) for 14 days then the rats were divided into 2 groups; one fed the 9% casein-control diet dry (. . . . .) the other fed the 9% casein-control diet in gel form (— — — —). At the points where each of the 2 lines again divide, each group was subdivided into 3 groups (8 rats/group) and fed the diets indicated on the graph. C = 9% casein control diet; L = 5% L-leucine diet; LIV = 5% L-Leucine diet plus additional isoleucine and valine (10 rats/group).

[5] 0.5% of the vitamin mixture in the diet provides the animals with 0.44% sucrose plus the following vitamins in mg/kg diet: thiamine·HCl, 5; riboflavin, 5; niacinamide, 25.0; Ca D-pantothenate, 20; pyridoxine·HCl, 5; folic acid, 0.5; menadione, 0.5; *d*-biotin, 0.2; vitamin B_{12} (0.1% mannitol), 30; ascorbic acid, 50 (added to help prevent thiamine destruction); vitamin E acetate (25% in a mixture of gelatin, sugar and starch), 400; vitamin A acetate and vitamin D_2 (325,000 USP units of vitamin A/g and 32,500 USP units of vitamin D_2/g in a mixture of gelatin, sugar and starch obtained from Hoffmann-LaRoche, Inc., Nutley, N. J.), 12.31.

[6] 1% of the vitamin mixture supplied the following per kg of diet: vitamin A acetate, 3000 IU; vitamin D_2, 300 IU; and (in milligrams) vitamin K (2 methyl-naphthoquinone), 5; thiamine·HCl, 10; riboflavin, 20; niacinamide, 50; ascorbic acid, 200; pyridoxine·HCl, 10; *p*-aminobenzoic acid, 100; biotin, 5; Ca pantothenate, 50; folic acid, 2; inositol, 200; and vitamin B_{12}, 0.05.

TABLE 2

Effects of various amino acid supplements on the growth of rats fed a low protein diet containing 5% L-leucine

Additions to 9% casein diet supplemented with 0.3% L-Met										Wt gain	Food intake
L-Leu	L-Ileu	L-Val	L-Thr	L-Trp	L-Phe	L-Tyr	L-His·HCl·H_2O	L-Lys·HCl	L-Arg·HCl		
%	%	%	%	%	%	%	%	%	%	*g/2 weeks*	*g/day*
—	—	—	—	—	—	—	—	—	—	42 ± 1 [1]	10.8
5	—	—	—	—	—	—	—	—	—	10 ± 1	6.1
5	0.3	0.3	—	—	—	—	—	—	—	35 ± 1	10.1
5	0.3	0.3	—	—	0.3	—	—	—	—	31 ± 3	8.8
5	0.3	0.3	—	0.1	0.3	—	—	—	—	29 ± 1	8.8
5	0.3	0.3	0.3	—	0.3	—	—	—	—	32 ± 2	8.5
5	0.3	0.3	0.3	0.1	0.3	—	—	—	—	56 ± 1	12.1
5	0.3	0.3	0.3	0.1	—	0.3	—	—	—	49 ± 3	—
5	0.3	0.3	0.3	0.1	0.3	—	0.15	—	—	58 ± 1	11.0
5	0.3	0.3	0.3	0.1	0.3	—	0.15	0.4	0.6	62 ± 2	11.8
Same as for group directly above + 0.5% Asn, 0.5% Gly, 0.5% Ala and 2.5% Glu										66 ± 1	11.7

[1] SE of mean.

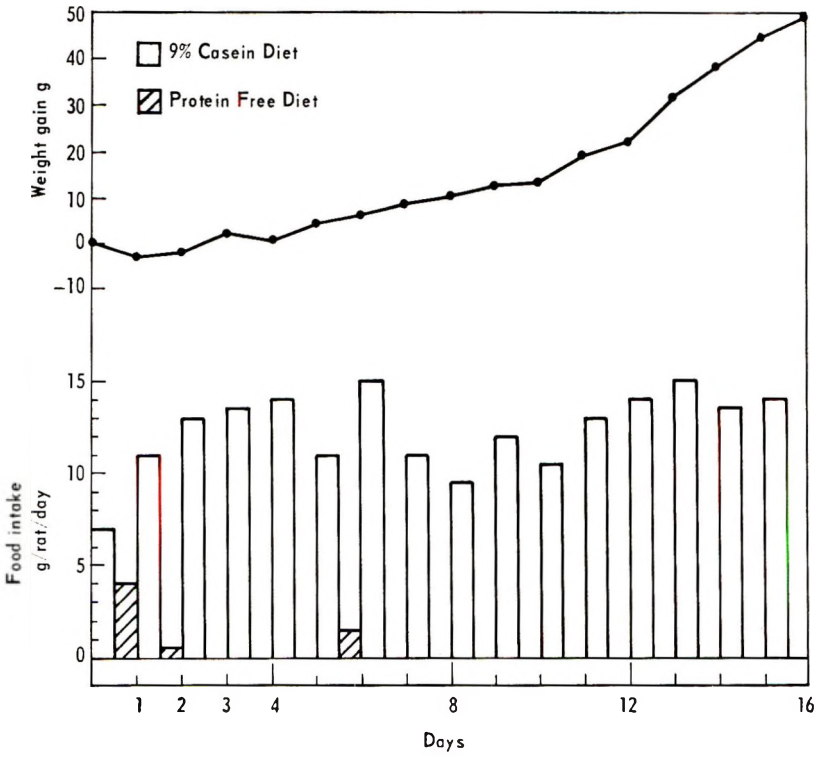

Fig. 2 Dietary choice of rats offered 9% casein control diet and a protein-free diet (10 rats/group).

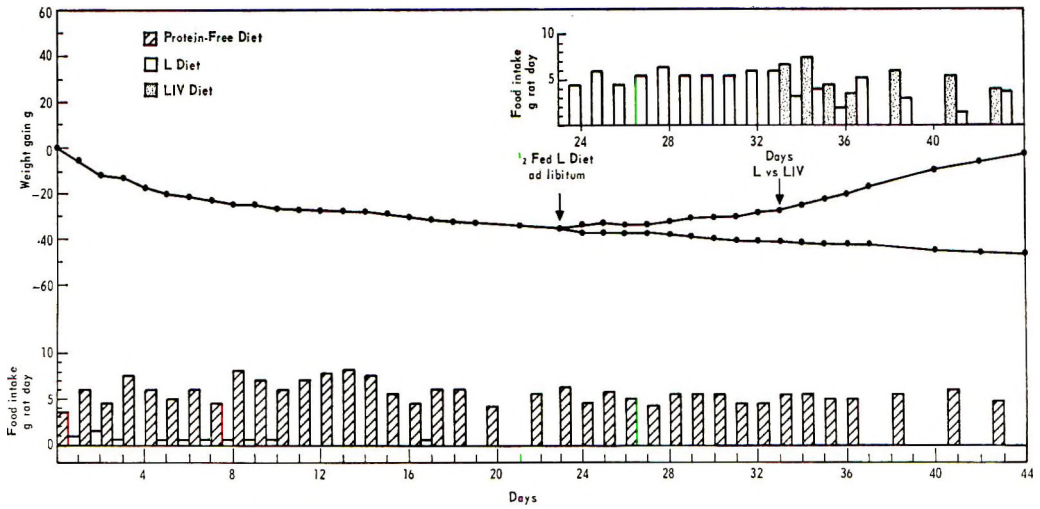

Fig. 3 Dietary choice of rats offered a 5% leucine diet (L) and a protein-free diet. LIV denotes the 5% leucine diet supplemented with 0.2% L-isoleucine and 0.2% L-valine (10 rats/group).

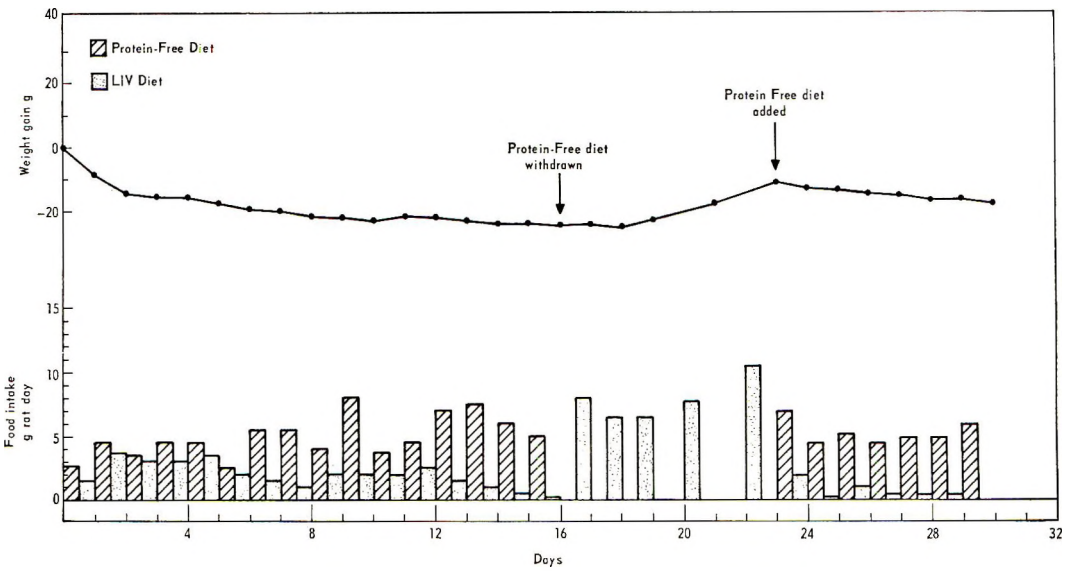

Fig. 4 Dietary choice of rats offered the high leucine diet supplemented with isoleucine and valine (LIV) and a protein-free diet (10 rats/group).

poured into a container to set. All of the agar gel diets contained approximately 50% moisture on a total wet-weight basis.

In the experiment in which the effect of feeding only a single meal daily was studied 50 rats (Holtzman strain) with an initial weight of 110 to 125 g were trained to eat their total daily food in a period of 2 hours. The training diet contained: (in per cent) casein, 25; salts (4), 4; corn oil, 5; vitamins (6),[7] 1; choline chloride, 0.2; and sucrose to make up to 100%. After 2 weeks on this regimen, the rats became adapted to the feeding program and were gaining weight. Half of the rats were then fed the basal experimental diet

[7] See footnote 5.

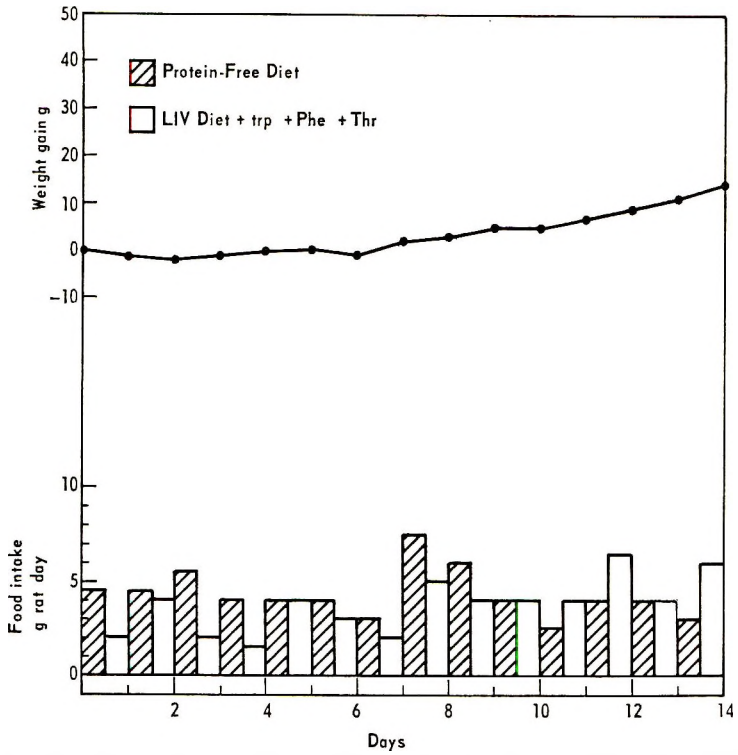

Fig. 5 Dietary choice of rats offered the high leucine diet supplemented with isoleucine, valine, tryptophan, phenylalanine and threonine and a protein-free diet (10 rats/group).

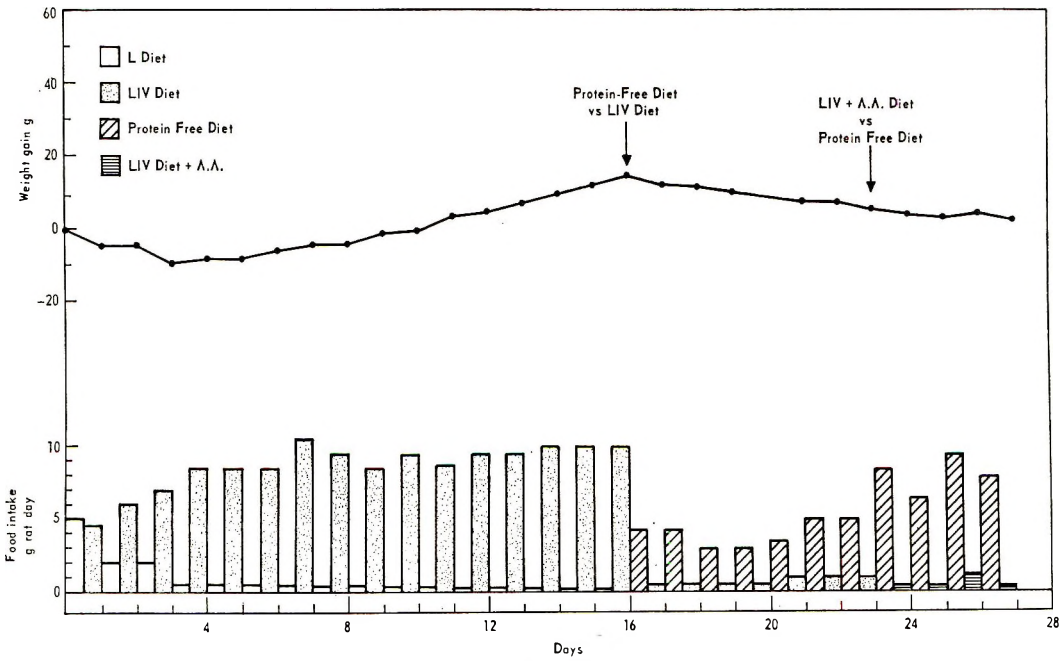

Fig. 6 Dietary choice of rats offered the high leucine diet (L) and a high leucine diet containing isoleucine and valine (LIV) (10 rats/group).

containing 9% casein, and the other half were fed the same basal diet but in the agar gel form. All the rats were offered their diet for only 2 hours a day. Again the rats were kept on this regimen for several days. Then each group of rats was divided into three smaller groups (8 rats to a group). Those that had been fed the dry basal diet previously were fed the three experimental diets, described above, in dry form; and those that had been fed the agar gel basal diet previously were fed the same 3 diets in gel form. All diets used in this experiment were supplemented with 0.3% DL-methionine.

RESULTS

Growth studies. The initial experiments were made to determine the effects of supplements of methionine and threonine, the limiting amino acids in casein, on the growth of rats fed a diet containing 9% of casein and 5% of L-leucine. The growth of the control group was stimulated by the supplement of methionine; that of the high leucine group was depressed; and that of the group receiving isoleucine and valine was slightly depressed (table 1). Growth of the control group was clearly stimulated by the supplement of threonine. The additional threonine did not markedly affect the growth of the groups fed either of the high leucine diets.

Since supplements of the two most limiting amino acids in the casein diet did not stimulate the growth of rats fed the high leucine diets, yet did stimulate the growth of the control groups, the effects of supplements of various other amino acids were examined. The results of an experiment which recapitulated several preliminary

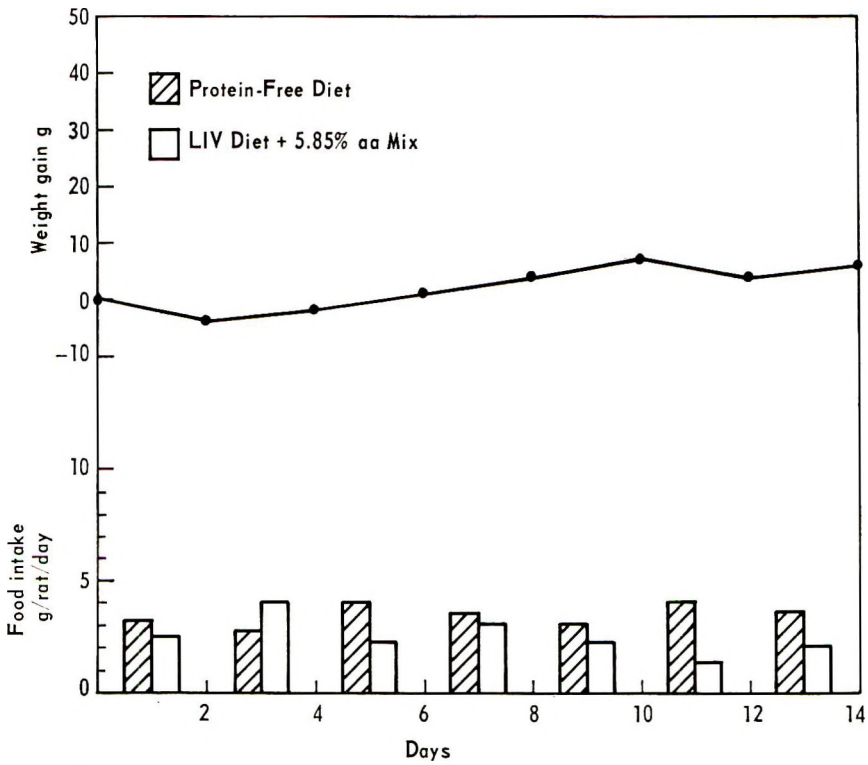

Fig. 7 Dietary choice of rats offered a protein-free diet and a high leucine diet supplemented with isoleucine and valine (LIV) plus the following amino acids: 0.3% L-phenylalanine, 0.3% L-threonine, 0.1% L-tryptophan, 0.15% L-histidine hydrochloride monohydrate, 0.4% lysine hydrochloride, 0.6% arginine hydrochloride, 0.5% asparagine, 0.5% glycine, 0.5% alanine, and 2.5% glutamic acid (10 rats/group).

trials [8] is presented in table 2. These show that phenylalanine, tryptophan and threonine must all be included with isoleucine and valine to stimulate further the growth of rats receiving 5% of L-leucine. Growth was improved, but not as much, by a similar mixture of amino acids in which tyrosine was substituted for phenylalanine. In earlier experiments (1) it had been shown that supplements of tryptophan and threonine either alone or together were ineffective.

Observations on animals fed for only 2 hours daily. The results of the experiment in which the rats were fed for only 2 hours a day are shown in figure 1. Each of the groups fed the agar-gel diets gained weight more rapidly than the comparable group fed the dry diet; however, both of the groups fed the high leucine diets gained less weight than their respective controls. The group fed the dry, high leucine diet did not show the usual adaption noted after 4 to 7 days with rats fed this diet ad libitum; however, the group fed the agar-gel high leucine diet did adapt and began to gain weight after a few days. As with rats fed ad libitum the groups fed the high leucine diet with additional isoleucine and valine gained more weight than the high leucine groups but not as much as the control groups.

Food selection studies. The results presented in this section are representative of at least two and usually three experiments in which similar results were obtained.

[8] Tannous, R., Ph.D. Thesis, Massachusetts Institute of Technology, 1964.

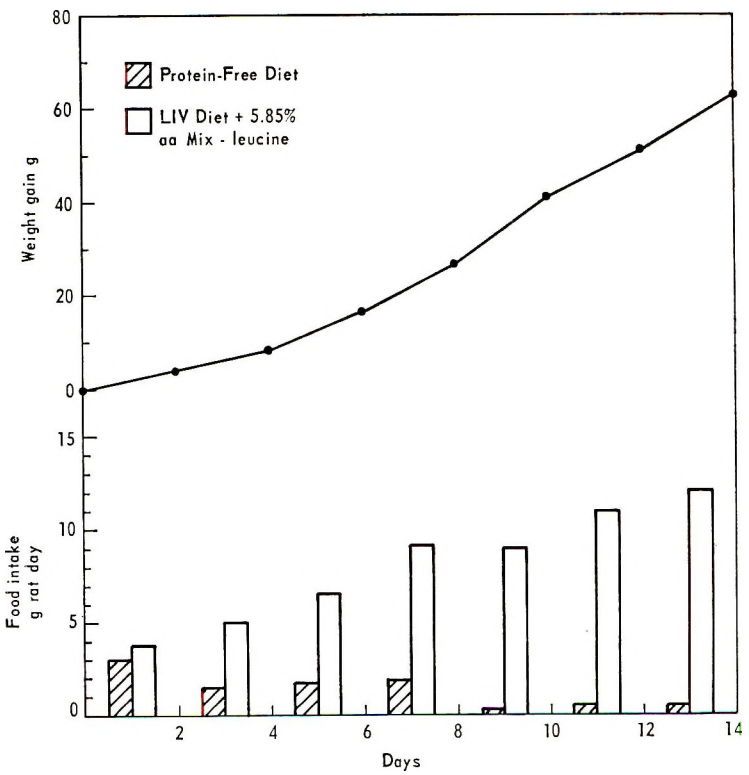

Fig. 8 Dietary choice of rats offered a protein-free diet and a high leucine diet supplemented with isoleucine and valine (LIV) plus the following L-amino acids: 0.44% arginine hydrochloride, 0.14% histidine hydrochloride monohydrate, 0.27% isoleucine, 0.47% lysine hydrochloride, 0.27% methionine, 0.38% phenylalanine, 0.27% threonine, 0.06% tryptophan, 0.27% valine, 0.11% alanine, 0.11% aspartic acid, 1.15% glutamic acid, 0.76% glycine, 0.11% proline, 0.11% cystine, 0.11% serine, 0.11% tyrosine, 0.20% asparagine and 0.49% sodium acetate (10 rats/group).

When rats were offered an ad libitum choice between the 9% casein control diet and a protein-free diet they almost invariably selected the control diet (fig. 2). When they were offered a choice between the high leucine diet and a protein-free diet they selected the protein-free diet and lost weight for 44 days (fig. 3) even though the high leucine diet would support a slow rate of growth. The ability of the high leucine diet to support growth is illustrated by the increase in weight observed for half of the rats from day 23 to day 33 when they were offered only the high leucine diet (fig. 3). On day 33 when these rats were offered a choice between the high leucine (L) diet and the high leucine diet supplemented with isoleucine and valine (LIV), they ate more of the LIV than of the L diet and grew more rapidly.

Rats offered a choice between the LIV diet which will support a fairly good rate of growth and a protein-free diet on which they cannot survive selected largely th' protein-free diet and lost weight for 1 days (fig. 4). From day 16 to day 23 the were fed the LIV diet ad libitum an gained 15 g. Thereafter, when they were again offered a choice between the LIV and protein-free diets, they ate largely the protein-free diet and lost weight. In another experiment only one of 10 animals selected the LIV diet and continued to eat it throughout the 14 days. When tryptophan, threonine and phenylalanine were added to the LIV diet to improve it (see table 2) and rats were offered a choice between this diet and a protein-free diet, although on the average they ate enough of the LIV diet to gain slowly (fig. 5), six

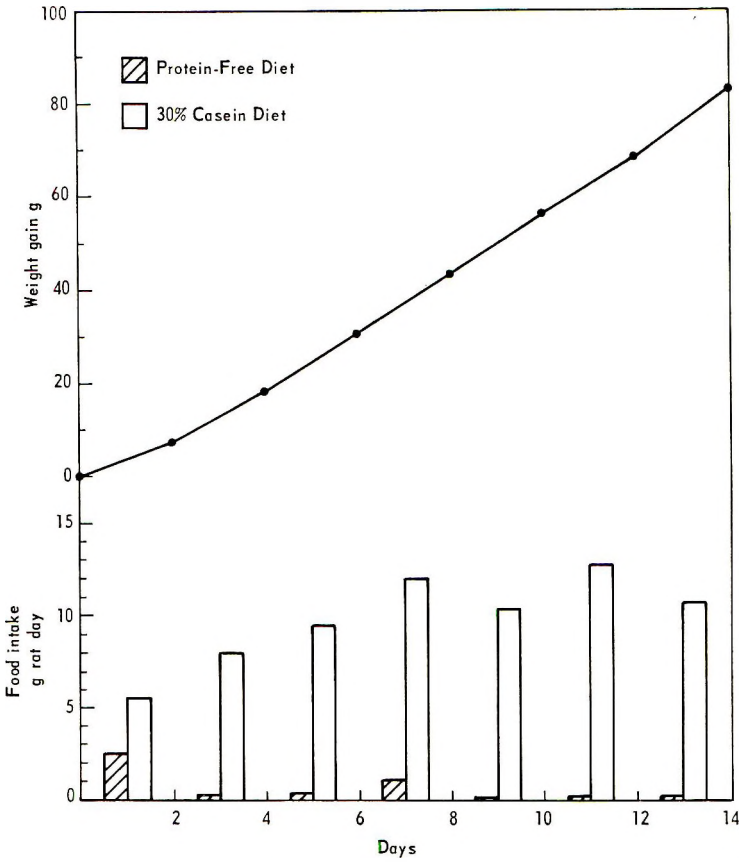

Fig. 9 Dietary choice of rats offered a protein-free diet and a 30% casein diet (10 rats/group).

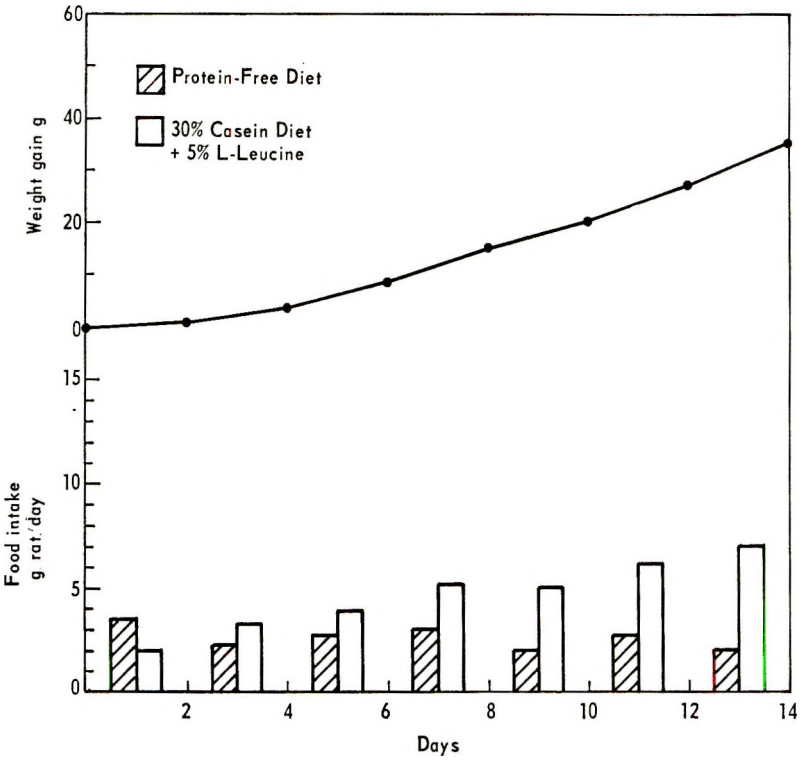

Fig. 10 Dietary choice of rats offered a protein-free diet and a 30% casein diet containing 5% L-leucine (10 rats/group).

of ten animals ate a higher proportion of the protein-free diet than of the LIV diet over the 14-day period.

In another trial rats were given a choice between the L and LIV diets. Results similar to those summarized in figure 6 were obtained in four separate experiments. After the first day there was a clear preference for the LIV diet. Nevertheless, when on day 16 a protein-free diet was substituted for the L diet, the animals that had been eating the LIV diet and growing well rejected it, ate very largely the protein-free diet and lost weight for a week. A more complete amino acid mix containing tryptophan, threonine and phenylalanine was then added to the LIV diet but the animals still continued to select the protein-free diet.

The failure of a high percentage of animals to select the LIV diet supplemented with tryptophan, phenylalanine and threonine (a diet that when fed ad libitum supported a good rate of growth, table 2) over the protein-free diet was unexpected, and hence a further trial was run in which rats were offered a choice between a protein-free diet and the diet that supported the most rapid rate of growth in the ad libitum feeding experiments (table 2). Again the animals showed a preference for the protein-free diet, eight of the ten eating mainly the protein-free diet throughout the 14-day trial (fig. 7). The mixture of amino acids included in this diet contained a large amount of glutamic acid [9] and hence, to determine whether this or the large total amount of free amino acids was influencing food selection, a complete mixture of amino acids, except for leucine which was already present in great excess, was substituted for the mixture of amino acids included in the LIV diet used in the experiment described in figure 7. The rats that were offered a choice between this diet and the protein-free diet selected the

[9] See figure legends for composition of the amino acid mixes.

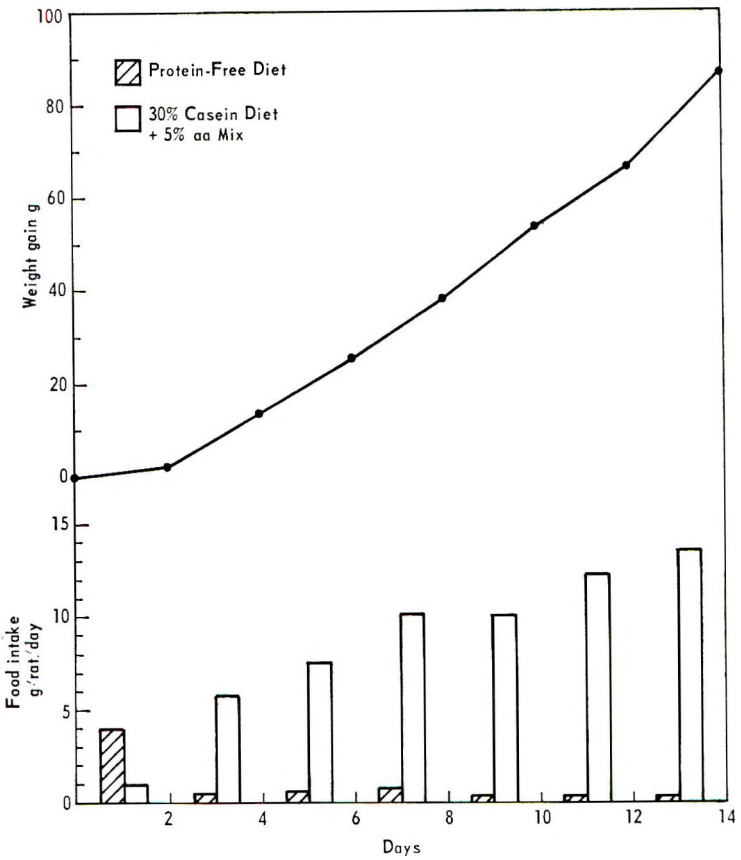

Fig. 11 Dietary choice of rats offered a protein-free diet and a 30% casein diet containing 5% amino acids. The amino acids were in the same proportions as in the mixture described in the legend for figure 8 (10 rats/group).

nutritionally superior diet and grew well. Only one of 10 rats showed a preference for the protein-free diet (fig. 8).

To get some indication of the extent to which diet selection was influenced by the addition of free amino acids independently of the nutritive value of the diet a trial was run in which rats were given a choice between a protein-free diet and a 30% casein diet, the 30% casein diet with 5% of L-leucine added or the 30% casein diet with 5% of a well-balanced amino acid mixture [10] added. The results are presented in figures 9, 10 and 11. Only rats offered a choice between the protein-free diet and the high leucine diet ate an appreciable quantity of the protein-free diet. Four of the 10 animals offered this choice ate a preponderance of the protein-free diet. Rats in the other 2 groups rejected the protein-free diet almost completely after the first day.

DISCUSSION

Prevention of growth depression due to excess leucine. The growth depression due to a large dietary excess of leucine involves amino acid interrelationships that are more complex than leucine-isoleucine and valine antagonism. Supplements of isoleucine and valine, which should counteract leucine-isoleucine and valine antagonism do not completely prevent the growth depression; threonine, tryptophan and phenylalanine are all required in addition to isoleucine and valine. Threonine is the growth-limiting amino acid in the basal diet, and hence the addition of amino

[10] See footnote 9.

acids other than threonine may create an amino acid imbalance involving threonine; whether the addition of leucine, isoleucine and valine would create an imbalance involving tryptophan and phenylalanine as well is problematical. If it were simply a matter of imbalance, some response at least would be expected to a supplement of threonine alone since it is more limiting than tryptophan or phenylalanine. It is possible that there is some more direct "antagonism" between excess leucine and tryptophan and phenylalanine. A leucine-tryptophan "antagonism" has been postulated (7) and some evidence of an antagonistic effect between leucine and aromatic amino acids has been reported (8, 9). Excess leucine was found to reduce plasma and muscle-free tyrosine and phenylalanine (10).

Two-hour feeding regimen. For certain metabolic studies it is useful to feed rats only once a day so that all animals will eat at the same time and the zero time can be synchronized (9). It is therefore necessary to show whether this type of feeding regimen may affect the nutritional response. In the present study an excess of leucine depressed the growth of rats fed for only 2 hours daily just as it does the growth of rats fed ad libitum.

Feeding the diets in gel form resulted in an improvement of growth. Water is known to improve protein utilization (11), particularly when protein is limiting in the diet. In the present experiments there was an increase in food intake when the diet was fed in gel form and this appears to be the major reason for the improvement of growth.

Food selection studies. The food selection studies provide further examples of conditions in which the rat will consistently select a protein-free diet in preference to a diet that will support growth (3). The selection of a protein-free diet over the high leucine diet is perhaps comparable to previous observations in which rats selected a protein-free diet over an imbalanced diet because the food intake of rats fed either the high leucine or the imbalanced diet is depressed. It was unexpected that the rat showed such a complete preference for the protein-free diet over the high leucine diet that was improved by the addition of isoleucine and valine. Even when the high leucine diet was further improved with supplements of tryptophan, threonine and phenylalanine to give a diet that would support more rapid growth than the control diet, at least half the rats still showed a preference for the protein-free diet for 2 weeks. However, rats showed a complete preference for the LIV diet without additional supplements over the L diet and for the LIV diet supplemented with a complete mixture of amino acids [11] over the protein-free diet. Yet a diet that supported about the same rate of growth but contained larger amounts of a few other amino acids was rejected in favor of the protein-free diet.

The response of reduced food intake when 5% leucine is included in a low protein diet is a very strong response. Krauss and Mayer (12) reported that the food intake of hyperphagic rats with lesions in the ventro-medial nuclei, was reduced when they were fed a high leucine diet. The mechanism, therefore, whereby the rat responds so strongly to a high leucine diet, awaits elucidation.

It is difficult to unravel the factors that influenced food selection in these experiments. There are obviously choices both for and against higher nutritional value. If it is valid to assume that, all other things being equal, a diet of higher nutritive value will be selected over one of poorer nutritive value because it promotes a greater feeling of well-being, it then becomes necessary to attribute selection of a nutritionally inferior diet over a nutritionally superior one to some unapparent physiological effects of the diets.

ACKNOWLDEGMENT

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[11] See footnote 9.

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Selenium-responsive Myopathies of Myocardium and of Smooth Muscle in the Young Poult [1]

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ABSTRACT Poor growth, mortality and myopathies of the heart and gizzard have been produced experimentally in young poults fed a practical-type diet containing all nutrients previously shown to be required by the poult except for supplemental vitamin E and methionine. The corn and soybean meal used in this diet were obtained from geographical areas where the soil is known to be low in selenium. Addition of vitamin E and methionine to the diet improved growth but gizzard myopathy was not prevented and maximal growth was not achieved until the diet also was supplemented with at least 0.1 ppm of selenium as sodium selenite. Under the conditions of these experiments, therefore, the selenium requirement in a practical-type diet depended to some extent upon the amount of vitamin E or methionine supplementation; it ranged from approximately 0.18 ppm in the presence of vitamin E to approximately 0.28 ppm of selenium in the absence of added vitamin E. Although myopathy of the skeletal (pectoral) muscle was not observed grossly, necropsy studies revealed histological changes in skeletal muscle typical of Zenker's degeneration. The order of prominence of the "selenium-responsive" diseases of the young poult appear to be: myopathy, first of the smooth muscle (gizzard); second, of the myocardium; and third, of the skeletal muscle. Selenium appears to be the primary nutritional factor required. Vitamin E is of less importance and sulfur amino acids are completely ineffective in prevention of these myopathies in poults.

Myopathies associated with vitamin E deficiency have been shown to differ widely among various species of animals. Vitamin E-deficient chicks also deficient in sulfur amino acids exhibit a severe myopathy of the skeletal muscles, especially the pectoral muscle, with changes in smooth or cardiac muscle tissues occurring only in rare instances (1). In lambs and calves both the skeletal and cardiac muscles are affected; no observations have been made of myopathies of the smooth muscle in these species.

Each of the three primary vitamin E deficiency diseases in chicks — encephalomalacia, exudative diathesis and nutritional muscular dystrophy — can be prevented by dietary supplements other than vitamin E. Encephalomalacia has been shown (2–5) to be prevented by synthetic antioxidants. The complete prevention of exudative diathesis in chicks by as little as 0.08 mg of selenium/kg of diet has been adequately demonstrated (6–9). Several groups of workers (10–12) have shown the effectiveness of sulfur amino acids in preventing nutritional muscular dystrophy (myopathy of skeletal muscle) in the chick. Hathcock and Scott (13) further showed that when the metabolic conversion of methionine to cysteine was blocked by the feeding of high levels of transmethylation metabolites, dietary methionine did not prevent dystrophy. These workers also found (unpublished) that the feeding of cholic acid to vitamin E-deficient chicks markedly increases the severity of nutritional muscular dystrophy, presumably by channeling cysteine out of the total cysteine-cystine metabolic pool for synthesis of taurocholate. In the prevention of nutritional muscular dystrophy in chicks, vitamin E metabolism must be related in some unknown way to the metabolism of cystine.

Although there are still many unanswered questions regarding the causes and basic mechanism involved in the vitamin E deficiency diseases in the chick, the order of occurrence of these diseases under

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deficiency conditions and the methods of preventing each are now well-established.

Vitamin E deficiency diseases in turkeys, however, are not as well understood. Gizzard myopathy in turkey poults was originally reported by Jungherr and Pappenheimer (14) to be the most characteristic, if not the only vitamin E deficiency symptom in turkeys. Since 1957, however, several groups of workers have shown that under appropriate conditions vitamin E-deficient poults may also show exudative diathesis (15) or nutritional muscular dystrophy (myopathy of skeletal muscle)[3] or both, as well as severe myopathies of the smooth muscle (gizzard) and the cardiac muscle. Ferguson et al. (16) reported nutritional myopathy of skeletal muscle in turkey poults receiving diets containing 10% ethyl linoleate in which the methionine and arginine levels were varied.

Walters and Jensen (17) reported nutritional myopathies of skeletal, gizzard and heart muscles in 4-week-old poults that received a vitamin E-deficient, selenium-low diet containing Torula yeast as the source of protein. These workers also noted the development of severe pericardial exudates and increased levels of glutamic-oxaloacetic transaminase. They reported that addition of the sulfur-containing amino acids to the diet had no beneficial effect on these myopathies, whereas selenium as sodium selenite was completely effective in prevention of all symptoms.

Interest in the selenium-responsive nutritional disease of poults was rekindled during the summer of 1964 when several commercial flocks of young turkeys in southwestern Ohio were found to grow poorly and suffer a high mortality rate with no overt signs of disease or nutritional deficiency except for a severe hyaline degeneration of the gizzard musculature. Upon investigation, it was found that both the corn and the soybean meal used in the turkey diets was produced locally in southern Ohio or Indiana on soils found by Muth and Allaway (18) to be very low in selenium and to produce forages so low in selenium that they fail to prevent white muscle disease in lambs and calves. Poults showing myopathy of the gizzard were found in 4 flocks totaling about 15,000 turkeys.

The case history of this outbreak was described by Bruins[4] as follows: "All poults were of a late hatch from a breeding flock located in northern Indiana. Histologic examinations of the affected gizzards by Dr. Frank Mitchell of Pitman-Moore Laboratories, Indianapolis, Indiana, revealed 'extensive acute coagulation necrosis and fibrous tissue replacement involving musculature of the gizzard.' Mitchell observed that he had 'not previously encountered cases similar to this among turkeys; however, coagulation necrosis of muscle fibers is a principal pathologic feature of vitamin E deficiency as it occurs in sheep, cattle, and other species.' Similar histopathology also was observed in the myocardium of poults from one of the turkey flocks. Mortality to 6 weeks of age ranged from 14 to 30% in three of the four flocks, and was 65% in the fourth flock which also was afflicted with chronic respiratory disease."

The experiments described in the present report were conducted to characterize the gizzard and cardiac myopathies, pericardial exudates, and myopathy of the skeletal muscle in the poult and to determine the influence of *d*-α-tocopheryl acetate, selenium, and the sulfur-containing amino acids on these myopathies. It was considered desirable to determine, if possible, the order in which these nutritional disorders occur in poults, and how the diseases respond to various dietary treatments.

Experiments were conducted using both a practical-type turkey diet composed of ingredients from selenium-low geographic areas and a semipurified diet of very low selenium content, to determine the selenium requirement for prevention of the myopathies of both the smooth muscle (gizzard) and the myocardium, as well as the requirements for normal growth and prevention of mortality.

Extensive histopathological studies were made of gizzard and heart tissues from selenium-deficient poults in an effort to

[3] Ferguson, T. M., and J. R. Couch 1961 Muscular dystrophy in turkeys. Federation Proc., 20: 297 (abstract).

[4] Bruins, H. W., personal communication.

more adequately characterize the deficiency disease.

EXPERIMENTAL

Replicate lots of 16 Broad Breasted White male poults were used in each of two experiments conducted with turkey diets composed of practical ingredients (corn and soybean meal) of known selenium content. An additional experiment was conducted with replicate lots of Broad Breasted White male poults fed a semipurified diet low in selenium, vitamin E and sulfur amino acids. The poults were housed in electrically heated, thermostatically controlled, wire-floor battery brooders. Feed and demineralized water were supplied ad libitum in stainless steel equipment.

The composition of the basal diet (A) used in the first 2 experiments is presented in table 1. In the first experiment the diet was supplemented with all nutrients known to be required by turkeys, except for vitamin E, selenium and methionine. In the second experiment, the same ration was used except for the addition of *d*-α-tocopheryl acetate at a level of 11 IU of vitamin E activity/kg of diet and 0.1% methionine hydroxy analogue. The basal diet was fed alone and supplemented with graded levels of selenium as sodium selenite. The levels ranged from 0.05 to 0.4 mg of added selenium/kg of diet. In experiment 1 the basal diet also was supplemented with 0.1% methionine alone and together with 0.1 ppm of selenium and with 11 IU of vitamin E/kg of diet alone and together with 0.1 ppm of selenium. As a further part of the experiment, the soybean meal used in basal diet A was replaced by soybean meal obtained from Sioux City, Iowa, where the corn and soybean crops are known to contain relatively higher amounts of selenium than that present in the corn and soybeans raised in the selenium-low areas of southwestern Ohio, southern Indiana, parts of New York State and many other localities. Analysis of the corn and soybean meal used in basal diet A showed selenium values of 0.06 and 0.1 ppm, respectively. Basal diet A was shown by analysis to contain the anticipated level of 0.08 ppm of selenium. The soybean meal used in basal diet B contained 1.04 ppm of selenium.

The basal, semipurified diet for the third experiment consisted of Torula yeast, isolated soya protein, selenium-low soybean oil meal and sucrose, as shown in table 2.

Daily records of mortality were maintained, and all dead poults were examined for myopathy of the skeletal and gizzard muscle as well as for pericardial transudates. At the end of the 5-week experimental periods all poults were killed and examined for deficiency symptoms. The severity of both skeletal and gizzard myopathy was scored on a scale ranging from zero for the absence of myopathy to 4 for

TABLE 1

Low selenium turkey starter diet (basal diet A)

	%
Low selenium corn (Cornell Mt. Pleasant)	39.165
Vitamin E-free lard	3.5
Low selenium soybean meal (Lawrenceburg, Indiana)	52.5
Dicalcium phosphate (USP, $2H_2O$)	3.25
$CaCO_3$, reagent grade	1.0
Manganese sulfate, CP	0.025
Zinc oxide, CP	0.0125
Salt, CP	0.25
Choline chloride, 70%	0.07
Vitamin mixture [1]	0.215

[1] Vitamin mix contributed the following levels per 100 kg of diet: (in IU) stabilized vitamin A, 1,100,000; vitamin D_3, 198,000; (in g) menadione sodium bisulfite, 0.45; riboflavin, 0.45; nicotinic acid, 4.5; Ca pantothenate, 3.3; folic acid, 0.22; pyridoxine, 0.45; vitamin B_{12}, 0.002; and zinc bacitracin, 5.5.

TABLE 2

Composition of semipurified basal diet

	%
Soybean meal	20.00
Isolated soybean protein	10.00
Torula yeast	15.00
Sucrose	39.20
Stripped lard	9.0
Glycine	0.5
Mineral mix [1]	4.947
Vitamin mix [2]	1.0
Choline chloride (70%)	0.35
Ethoxyquin	0.003

[1] Mineral mix contributed the following levels per 100 kg of diet: (in grams) $CaHPO_4 \cdot 2H_2O$, 952; $CaCO_3$, 2154; NaCl, 700; KH_2PO_4, 1500; $MgSO_4 \cdot 7H_2O$, 500; $MnSO_4 \cdot H_2O$, 25; $FeSO_4 \cdot 7H_2O$, 20; ZnO, 9.8; $CuSO_4 \cdot 5H_2O$, 2; KI, 0.5; and $NaMoO_4 \cdot 5H_2O$, 0.4.

[2] Vitamin mix contributed the following levels per 100 kg of diet: (in IU) vitamin A, 1,300,000; vitamin D_3, 193,000; (in grams) menadione sodium bisulfite, 0.4; riboflavin, 1; thiamine, 1; pyridoxine, 1; nicotinic acid, 8.8; Ca pantothenate, 2.2; folic acid, 0.2; biotin, 0.022; and vitamin B_{12}, 0.002.

maximal severity. Pericardial transudates were scored only as present or absent.

Necropsy. Poults were chosen periodically at random from the various treatment groups in experiment 1 for necropsy. Gizzard, pectoral muscle, heart and liver were fixed in Bouin's solution. Paraffin embedding and sectioning at 6 μ were used. Tissues were stained with hematoxylin and eosin, van Gieson's connective tissue stain, Ladewig's modification of Mallory's trichrome stain and, in some cases, with phloxin.

RESULTS

Results with practical-type diets. Experiment 1. The results of the first experiment with the practical diet (table 3) show that the poults receiving the basal diet grew poorly, suffered a high rate of mortality and a 97% incidence of myopathy of the gizzard with an average severity score of 2.7. Addition of 0.05 mg selenium (as sodium selenite)/kg of diet caused a marked improvement in growth, reduced the mortality and the severity of the gizzard myopathy, but did not prevent it. Myopathy of the gizzard was prevented by 0.2 ppm added selenium but not by the addition of 0.1 ppm. Thus, the selenium requirement for prevention of myopathy of the gizzard under the conditions of this experiment appeared to be greater than the selenium requirement for maximal growth, and was approximately 0.18 to 0.28 ppm (0.18–0.28 mg of total selenium/kg of diet).

Addition of methionine improved growth but caused only a slight improvement in the incidence of gizzard myopathy. The addition of vitamin E alone also improved growth but caused only a small improvement in severity of the gizzard myopathy. The addition of 0.1 ppm of selenium, together with either 11 IU vitamin E/kg of diet or 0.1% methionine, completely prevented the gizzard myopathy. The addition of 0.1% methionine and 0.1 ppm of selenium improved growth by approximately 100 g above that obtained with either methionine or selenium alone. Basal diet B, which contained by analysis 0.37 ppm of selenium, produced the same rate of growth obtained with basal diet A supplemented with selenium at 0.05 to 0.4 ppm and completely prevented myopathy of the gizzard. Further addition of selenium to this diet at a level of 0.4 ppm caused only a slight improvement in growth.

Mortality, in most cases, was due to the myopathy of the heart muscle, which resulted in severe pericardial exudates.

No gross evidence of myopathy of skeletal muscles was obtained, but necropsy studies, presented in detail later in this report, showed the presence of slight-to-moderate Zenker's degeneration of these muscle fibers in poults receiving the basal

TABLE 3

Effectiveness of selenium for growth and prevention of gizzard myopathy in young poults (exp. 1)

Lot no.	Treatment	Average weights, 4 weeks	Gizzard myopathy		Mortality
			Incidence	Severity score (0–4)	
		g	%		%
1	Basal diet A	465(435–495) [1]	97	2.7	36
2	+ Se, 0.05 ppm	591(586–595)	50	0.6	22
3	+ Se, 0.1 ppm	584(568–597)	19	0.3	9
4	+ Se, 0.2 ppm	605(576–635)	0	0	0
5	+ Se, 0.4 ppm	589(562–616)	0	0	0
6	+ 0.1 DL-methionine	584(552–616)	85	1.5	25
7	+ 0.1% DL-methionine + Se, 0.1 ppm	683(660–705)	0	0	3
8	+ vitamin E, 11 IU/kg	560(537–582)	55	1.0	3
9	+ vitamin E, 11 IU/kg + Se, 0.1 ppm	569(565–573)	0	0	3
10	Basal diet B	584(580–588)	0	0	12
11	+ Se, 0.4 ppm	611(602–620)	0	0	3

[1] Numbers in parentheses show averages for individual lots of 16 male Broad Breasted White poults.

diet and the lowest level of supplementary selenium.

Experiment 2. The second experiment was conducted with the same basal diet except for the addition of 0.1% methionine hydroxy analogue and vitamin E at a level of 11 IU/kg of basal diet. The basal, selenium-low diet was fed alone and supplemented with the same graded levels of selenium (as sodium selenite) used in experiment 1. The results of the experiment (table 4) show that although no mortality occurred in poults receiving the basal diet, there was about a 25% incidence of gizzard myopathy. The severity score was relatively low, however, indicating that in most instances less than one-fourth of the total musculature was affected, even in those poults showing the myopathy. The addition of 0.05 ppm of selenium did not completely prevent gizzard myopathy, but the addition of 0.1 ppm was completely effective, thereby confirming the results of the first experiment which indicated that 0.1 ppm of added selenium or a total of approximately 0.18 ppm represents the selenium requirement for prevention of gizzard myopathy in the presence of 0.1% methionine and added vitamin E at a level of 11 IU/kg of diet. No gizzard myopathy occurred in any of the poults receiving basal diet B.

In an attempt to determine whether addition of arsenicals to a basal, low selenium diet increases the severity of selenium deficiency, triplicate lots of poults in experiment 2 were fed the basal diet plus a combination of arsanilic acid, 90 mg/kg, and *p*-ureidobenzenearsonic acid (37.5% pure), 1 g/kg of diet. The addition of these arsenicals did not appear to increase the incidence or severity of gizzard myopathy.

Experiment 3. Results with the semi-purified diet. In all poults not receiving selenium, mortality was high from day 16 to the end of the experiment. Necropsy showed severe myopathy of the gizzard muscle with scores averaging 3.5 to 4.0 and severe pericardial transudates. The cause of death in these poults did not appear to be due to myopathy of the gizzard but rather to the loss of blood fluids from the heart. The transudative fluid usually ranged in color from clear tc a deep red. In a few cases, however, the transudates appeared as a white gelatinous substance completely encompassing the heart. Only one poult was observed with generalized exudative diathesis similar to that commonly observed in chicks receiving vitamin E- and selenium-deficient diets.

The results on autopsy presented in table 5 showed that all poults except those receiving the diet containing selenium suffered from severe myopathy of the gizzard. Over 60% of all poults not receiving selenium showed pericardial transudates. No pectoral muscular dystrophy was noted in the poults receiving the basal diet or the basal diet plus selenium.

TABLE 4

Need for selenium for prevention of gizzard myopathy in poults receiving a practical-type diet supplemented with vitamin E (exp. Pr-2)

Treatment	Average weights, 4 weeks	Gizzard myopathy	
		Incidence	Severity score (0–4)
	g	%	
Basal diet A (vitamin E, 11 IU/kg + 0.1 methionine hydroxy analogue)	642(620–693) [1]	25	0.2
+ Se, 0.05 ppm	654(640–662)	25	0.09
+ Se 0.2 ppm	639(636–642)	0	0
+ Se, 0.4 ppm	655(650–665)	0	0
+ arsanilic acid, 90 mg/kg + *p*-ureidobenzenearsonic acid, 37% pure, 1 g/kg	604(595–623)	27	0.25
Basal diet B + vitamin E, 11 IU/kg	625(610–642)	0	0
+ Se, 0.4 ppm	571(560–582)	0	0

[1] Figures in parentheses show ranges of average weights for triplicate lots of 16 Broad Breasted White poults per lot except that duplicate lots of 16 poults were used for the last 2 treatments with basal diet B. Mortality was very low in all lots in this experiment.

TABLE 5

Complete effectiveness of selenium in prevention of myopathies of heart and gizzard in vitamin E-deficient poults

Treatments	Myopathy				
	Pericardial transudates	Growth		Pectoral muscle	
	Incidence	Severity score (0–4)	Incidence	Severity score (0–4)	Incidence
None (semipurified diet)	14/15	3.6	15/15	0	0/15
Selenium (as Na_2SeO_3), 1.0 mg/kg	0/17	0	0/17	0	0/17

TABLE 6

Summary of macroscopic and microscopic lesions in gizzard, pectoral and heart muscles, and liver (exp. Pr-1)

Diets	Age at autopsy	Lesions, severity scores (0–4)					
		Macroscopic		Microscopic			
		Gizzard	Pectoral	Gizzard	Pectoral	Heart	Liver
	weeks						
Basal Se-low diet	1(3) [1]	0	0	0	0	—	—
	2(2)	0.5	0	0.5	0.5	—	—
	2.5(2)	3	0	4	0.75	0	0
	3(2)	3	0	4	2	2	0
	4(4)	4	0	3	1	2	0
Basal + 0.05 ppm Se	2.5(1)	3	0	4	0.5	0	0
	4(2)	1	0	2	1	2	0
Basal + 0.4 ppm Se	1(2)	0	0	0	0	—	—
	2(2)	0	0	0	0	—	—
	3(3)	0	0	0	0	0	0
	4(1)	0	0	0	0	0	0
Basal + 0.1% DL-methionine	2(2)	2	0	2	0.5	—	—
	3(3)	3	0	3	0.5	3	0
	4(2)	3	0	2	1	2	0

[1] Numbers in parentheses indicate number of poults autopsied.

Results of necropsy

The degree of severity of macroscopic and microscopic lesions is summarized in table 6.

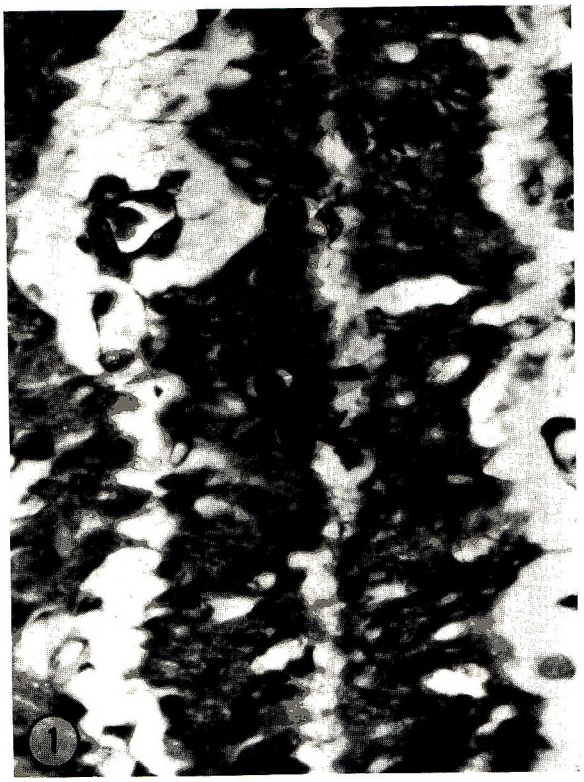
1. Macroscopic examination. a. Gizzard. In the acute stage of deficiency the cut surface was paler than normal, slightly bulging, and moist. Light yellowish areas gave a mottled appearance against the otherwise waxy cut surface. In advanced cases the thickness of the muscular wall was markedly reduced often to less than one-third normal size. The wall was firm. Haphazardly distributed fibrous scars traversed the tissue. The cut surface was dry, mottled gray and brown and not bulging. The mucosa was intact in all stages.

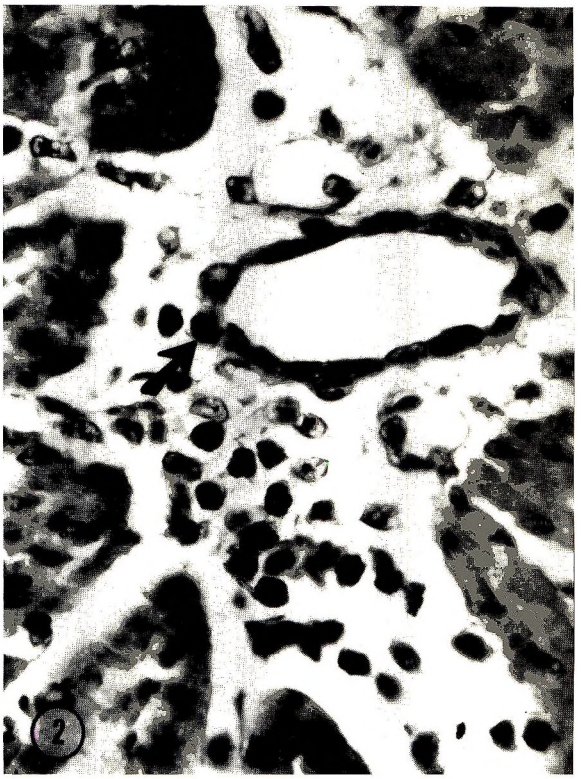
Fig. 1 Upper left: Gizzard. Acute Zenker's degeneration with hyaline bodies at arrows. Edema separating muscle bundles. (Basal diet, 2 weeks.) H & E, oil immersion. × 800.

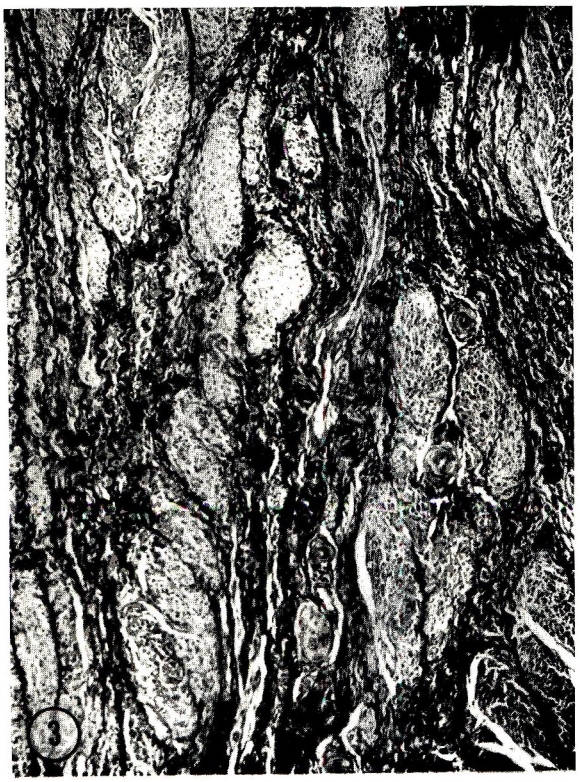
Fig. 2 Upper right: Gizzard. Intramuscular edema with heterophilic leukocytosis. Heterophilic leukocyte at arrow apparently migrating through vessel wall. (Basal diet, 3 weeks.) H & E, oil immersion. × 800.

Fig. 3 Lower left: Gizzard. Advanced loss of parenchyma. Severe fibrosis. (Basal diet, 4 weeks.) van Gieson. × 120.

Fig. 4 Lower right: Gizzard, normal. Same stain and magnification as in figure 3. (Basal diet + 0.4 ppm selenium, 4 weeks.)







b. Muscle. No gross lesions were observed. There was no inter- or perimuscular transudation.

c. Heart. Poults that died or were dying showed hemopericardium. This was apparently due to diffuse epicardial effusion but in cases with heart tamponade a rupture of the left atrium was observed. The cut surface indicated acute Zenker's degeneration. Chronic lesions were not recorded.

d. Liver. Gross lesions were not found.

2. *Microscopic examination.* a. Gizzard. The earliest lesion in the selenium-deficient poults was the appearance of hyaline bodies in the gizzard muscle cytoplasm. Eosinophilic, intensely phloxin-positive, structureless granules or, more commonly, large globules up to 15 μ in diameter appeared in cytoplasmic vacuoles. Characteristically the hyaline body did not fill the vacuole and an unstained halo was left surrounding the body (fig. 1). Vacuolation without hyaline bodies also was observed.

At this early stage there was also pronounced edema separating the degenerated muscle bundles (figs. 1–2). The edema stained poorly with hematoxylin and eosin, apparently due to low protein content. A prominent histologic feature was the presence of heterophilic leukocytes in this edema (fig. 2). These occurred as perivascular cuffings or diffusely throughout the edema. Practically no other leukocytes were found migrating, nor was there any erythrodiapedesis.

With increasing chronicity the loss of parenchyma became more accentuated. The muscle tissue simply shrank and the fibrous tissue thus became more densely packed (fig. 3; for comparable normal, see fig. 4). Fibrosis of the muscle bundles also occurred with thin, well-collagenized strands penetrating into the muscle. The distribution of the lesions was haphazard as shown in figure 5, but the innermost layer usually was spared.

Proliferation of muscle nuclei also was a prominent feature of the chronic lesions (fig. 6). Distribution of nuclei was haphazard; large areas often were occupied by aggregations of muscle nuclei, some of which were in mitosis (fig. 6), whereas in other fields fibrosis alone predominated the histologic picture (fig. 3).

Hyaline bodies were observed occasionally in gizzards of poults with the chronic lesion. Heterophilic leukocytosis, sometimes with perivascular cuffing, persisted in the chronic stage.

Fibrinoid degeneration of arteries was never observed.

b. Muscle. Changes in the pectoral muscle were less common and less severe than in the gizzard. The lesions consisted of acute Zenker's degeneration of the usual morphology and moderate intramuscular edema with mild to moderate heterophilic leukocytosis. No nuclear proliferation or fibrosis was observed.

c. Heart. The heart changes in the selenium-deficient poults consisted of an acute Zenker's degeneration with site of predilection in the atria. Associated with these changes were extensive intramural, subendo- and subepicardial edema and hemorrhage with heavy admixture of heterophilic leukocytes. Subepicardial hemorrhages occasionally caused rupture of the epicardium (figs. 7 and 8). No evidence of chronic lesions of the heart was found in the young poults even where chronic gizzard myopathy was observed.

d. Liver. No changes were observed.

DISCUSSION

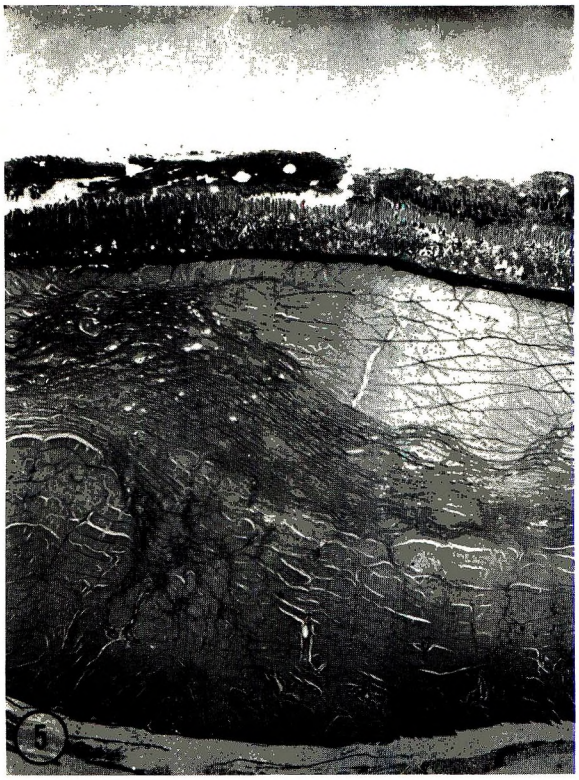
The results show that myopathy of the gizzard is the first symptom to appear in poults deficient in selenium and vitamin E. It is followed by myopathy of heart muscle, particularly of the atrium, resulting in

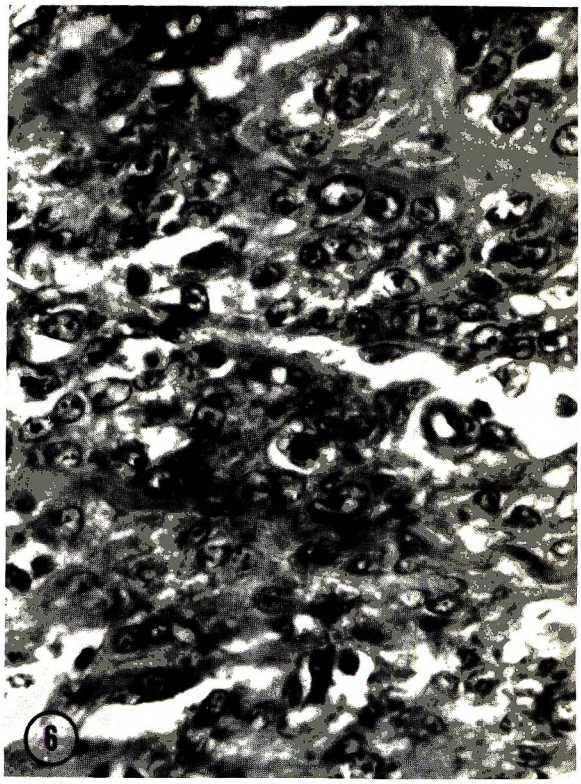
Fig. 5 Upper left: Gizzard. Severe fibrosis of haphazard distribution. (Basal diet, 4 weeks.) van Gieson. × 10.

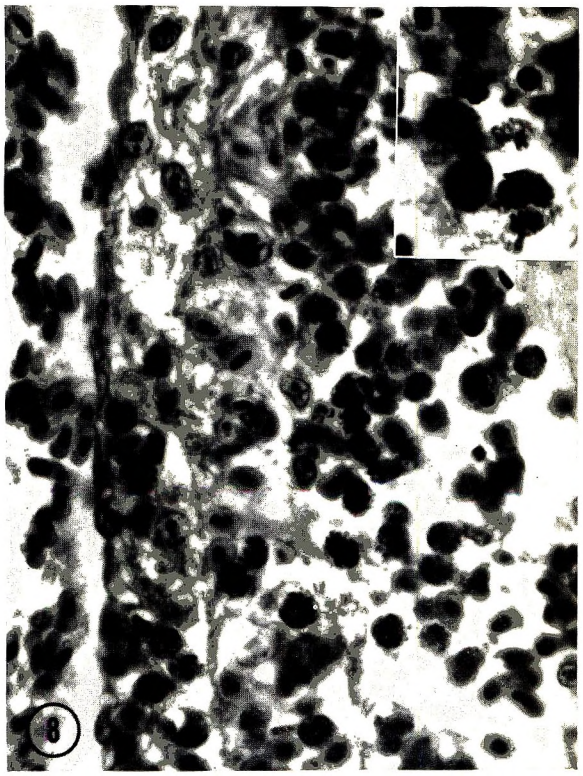
Fig. 6 Upper right: Gizzard. Pronounced proliferation of muscle nuclei. Nucleus just below center in mitosis. (Basal diet, 2 weeks.) H & E, oil immersion. × 800.

Fig. 7 Lower left: Heart, left atrium. Acute Zenker's degeneration with muscle bundles separated by edema and hemorrhages. (Basal diet, 3 weeks.) H & E. × 120.

Fig. 8 Lower right: Heart, left atrium. Erythrocytes in left portion of field are outside epicardium. Extensive subepicardial hemorrhage and edema with admixture of heterophilic leukocytes. H & E, oil immersion. × 800. Inserted in upper right corner: detail of heterophilic leukocytes. (Basal diet, 3 weeks.) H & E, oil immersion. × 1,150.







pericardial transudates which apparently cause death in a high percentage of the cases. If, however, the poults survive these two deficiency diseases, they may exhibit nutritional muscular dystrophy of the pectoral muscle between the fourth and fifth weeks of age.

The earliest histological expression of the vitamin E-selenium deficiency syndrome in poults is the appearance of so-called hyaline bodies in the gizzard and heart muscles. Hyaline bodies were originally described by Mallory (19) as pathognomic of alcoholic liver cirrhosis, but later were shown to occur in choline deficiency (20) as well as in certain fetal deaths and in death of aviators at high altitudes (21, 22). Hartroft (23) showed in electron micrographs that hyaline bodies consisted of conglutinated degenerate megamitochondria. Hyaline bodies also were shown to be the initial lesion in liver cells in experimentally induced nutritional liver necrosis in beef cattle (24). The demonstration of hyaline bodies in the gizzard and heart muscles in the present study may, therefore, be interpreted as an expression of cellular anoxia as a result of the vitamin E-selenium deficiency.

Selenium appears to be the primary factor concerned in the prevention of the entire syndrome in poults, since selenium was completely effective in preventing all symptoms, whereas vitamin E prevented pericardial exudates and skeletal muscle dystrophy but failed to entirely prevent myopathy of the gizzard. The results of these studies confirm those of Walter and Jensen (17) indicating that cystine is not primarily involved in the prevention of the nutritional myopathies associated with vitamin E and selenium deficiency in the poult.

The results of these studies, together with the studies on the quantitative selenium requirements for prevention of exudative diathesis in chicks (9), provide a probable reason for the fact that the basic, classical vitamin E-deficient diet 108 of Pappenheimer and Goettsch (25) prevented exudative diathesis and produced only encephalomalacia in chicks, and when used with turkey poults by Jungherr and Pappenheimer (14) produced only myopathy of the gizzard. These results would be expected if diet 108 contained more than 0.1 ppm of selenium (the amount required to prevent exudative diathesis in chicks), but less than 0.28 ppm of selenium (the amount required in absence of vitamin E to prevent myopathy of gizzard in poults).

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