Influence of Raw and Heated Soybean Meal and of Soybean Trypsin Inhibitors on the Activity of Certain Protein Catabolism Enzymes in Kidneys and Livers of Chicks and Rats

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ABSTRACT Enzymatic activities involved in protein metabolism — liver xanthine dehydrogenase, liver xanthine oxidase (EC 1.2.3.2), liver aspartate aminotransferase (EC 2.6.1.2), liver and kidney arginase (EC 3.5.3.1) – were studied in chicks and rats fed diets containing the following soybean meals as protein sources: 1) heated soybean meal (HSBM); 2) raw soybean meal (RSBM); 3) HSBM + RSBM (2:1); and 4) HSBM supplemented with soybean trypsin inhibitors. RSBM decreased significantly the xanthine dehydrogenase, xanthine oxidase and arginase activity as compared with HSBM, whereas aspartate aminotransferase and alanine aminotransferase were affected slightly. The HSBM + RSBM or HSBM + soybean trypsin inhibitors did not show any clear influence on enzymatic activities. Diets with 25% protein gave higher enzymatic activity than those of 15% and methionine supplementation had no significant effect. Good correlation was found between the means of xanthine dehydrogenase and arginase activities in chicks and between the means of these activities and growth rate of each group.

In view of the fact that raw soybean meal (RSBM) impairs growth of chicks, rats and other animals, investigations of certain stages in protein catabolism might shed light on the mechanisms involved in this phenomenon. When rats were fed RSBM diets, noteworthy decreases of liver xanthine oxidase (EC 1.2.3.2) and amino transferase (EC 2.6.1) activities were noted, compared with these activities in animals fed heated soybean meal (HSBM). The dependence of enzymatic activities in rats on the nutritive value of dietary protein has been studied by numerous investigators. Westerfeld et al. (1) reported a relationship between protein level in the diet and liver xanthine dehydrogenase activity in chicks. Good correlation was also found between the increase in body weight in chicks and liver xanthine dehydrogenase and kidney arginase (EC 3.5.3.1) activities, as well as clear dependence of these enzymatic activities upon level and nutritive value of the protein.[1] In the present investigation the effect of RSBM and HSBM diets and of HSBM diets supplemented with the Kunitz's crystalline soybean trypsin-inhibitor (CSBTI) (2) with the pure soybean trypsin- and α-chymotrypsin-inhibitor AA (3) were studied on certain enzymes from chicks and rats which are associated with protein catabolism in these animals. The effects of methionine supplementation and protein level in these diets were also examined.

Xanthine oxidase or xanthine dehydrogenase participate with a wide specificity in many oxidation reactions. As the birds are uricolitic animals, xanthine dehydrogenase activity controls the formation of uric acid, the main end product of protein catabolism. Arginase is a highly specific enzyme but its activity controls the intensity of urea synthesis — the additional end product of protein catabolism. It should be considered, however, that in chicks ornithine is not converted to citrulline (4) and therefore the function of arginase is probably limited only to metabolism of arginine and citrulline. The main arginase activity in chicks exists in the kidneys (5–7), whereas xanthine dehydrogenase activity is concentrated in the liver; therefore, kidney arginase and liver xanthine dehydrogenase assays were carried out. Aspartate and glutamate are known as NH_3 donors for urea synthesis. Since aspartate aminotransferase (EC 2.6.1.1) and alanine

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aminotransferase (EC 2.6.1.2) catalyze glutamate and aspartate synthesis, liver aspartate aminotransferase and alanine aminotransferase activities were determinted in order to evaluate glutamate and aspartate turnover.

MATERIALS AND METHODS

RSBM, HSBM, trypsin inhibitors AA and CSBTI, the experimental conditions and diets for growth trials 1, 2 and 3 are the same as described in an earlier publication by Gertler et al. (8), as follows.

Soybean meals and soybean trypsin inhibitors. Raw soybean meal (RSBM) was prepared by milling soybeans (Harosoy variety) and defatting the soybean flour by extraction with ethyl ether in a Soxhlet apparatus. The nitrogen content of RSBM was 7.95%.

Properly heated soybean meal (HSBM) [2] was a commercial product with a nitrogen content of 8.0%.

The trypsin inhibitor AA was prepared according to Birk et al. (3). Crystalline soybean trypsin inhibitor (CSBTI) was a commercial product.[3]

Feeding trials with chicks and rats. One-day-old male White Leghorn or New Hampshire × White Leghorn chicks were fed sorghum grains for 4 or 5 days. They were then weighed individually, distributed into groups of 10 or 20 chicks in one pen for each feed treatment and housed in electrically heated battery brooders with raised wire floors. The chicks were grown with an all-vegetable diet, the main protein source being the different experimental soybean meals, with or without added trypsin inhibitors. The composition of the experimental diet is shown in table 1. Food and water were given ad libitum and the trials were terminated after 7 to 10 days.

The amount of ingested food was determined for the group as a whole, the chicks were weighed individually, killed by rapid bleeding and certain organs were removed immediately for determination of weights and activities.

The following amounts of soybean trypsin inhibitors were added to the diets: 5.5 mg of inhibitor AA were added per gram of HSBM which corresponds to the calculated inhibitor AA content in RSBM; in several experiments 16.5 mg inhibitor AA were added per gram of HSBM, yielding a diet which contained the same in vitro total trypsin-inhibiting activity as a corresponding diet with RSBM as the protein source; and CSBTI was added in amounts corresponding to those of inhibitor AA on a weight basis.

Six litters of weanling male rats and 6 litters of weanling female rats, of an inbred Wistar strain were used for this feeding trial. Each litter included 4 males or 4 females. Twelve rats (6 males and 6 females, one animal from each litter) were taken for each feed treatment. They were housed in groups of six (one group of 6 males and one group of 6 females for each diet) in cages with wire floors in a room maintained at 25°. The trial was carried on for 2 weeks and the rats were fed all-vegetable diets containing 10% protein supplied by the four experimental soybean meal (RSBM, HSBM, RSBM + HSBM in the ratio of 1:2, and HSBM supplemented with 0.55% of inhibitor AA) as the only protein source. The composition of these diets is shown in table 2. Food and water were given ad libitum. The rats were weighed individually at the beginning and at the end of the trial and the amounts of

TABLE 1

Composition of experimental diet for chicks

	%
Soybean oil	3.0
α-Cellulose [1]	3.0
Ground oyster shells	1.5
Dicalcium phosphate	2.0
Mineral mixture [2]	0.5
Vitamin mixture [3]	0.2
Choline chloride	0.1
DL-Methionine [4] (98% pure)	0.6
Soybean protein source [5]	
Carbohydrate mixture [6] to total 100	+

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.

[2] Composition: (in percent) NaCl, 94.70; MnO, 3.36; KI, 0.06; $Cu(OH)_2$, 0.14; ZnO, 0.09; $FeCO_3 \cdot 2H_2O$, 0.33; Na_2MoO_4, 0.01; and Ca stearate to make 100.

[3] Composition per 2 g: (in milligrams) thiamine·HCl, 5; riboflavin, 5; niacin, 60; Ca pantothenate, 25; pyridoxine·HCl, 5; folic acid, 4; biotin, 0.2; menadione, 0.5; and tocopheryl acetate, 30; and vitamin B_{12}, 20 μg; vitamin D_3, 480 IU; and vitamin A, 5000 IU.

[4] In certain diets DL-methionine was omitted.

[5] Given to supply 17, 22 or 25% protein (6.25 × N) according to nitrogen content of various meals.

[6] Composed of equal amounts of dextrose and potato starch.

[2] Prepared at Izhar Oil Industry of Israel Ltd., by heating the defatted flakes at 80° to 100° for 20 minutes at 118° and drying at room temperature.

[3] Worthington Biochemical Corporation, Freehold, New Jersey.

TABLE 2
Composition of experimental diets for rats

	%
Dextrose	33.1
Potato starch	33.1
Soybean oil	8.0
α-Cellulose [1]	1.0
Salt mixture no. 2, USP	4.0
Vitamin mixture [2]	0.3
Choline chloride	0.2
DL-Methionine	0.3
Soybean protein source [3]	20.0

[1] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[2] Composition per 3 g: (in milligrams) thiamine·HCl, 10; riboflavin, 15; pyridoxine·HCl, 15; Ca pantothenate, 20; folic acid, 2; niacin, 30; biotin, 0.3; inositol, 100; p-aminobenzoic acid, 25; menadione, 4; tocopheryl acetate, 30; and vitamin B_{12}, 25 μg; vitamin D, 5000 IU; and vitamin A, 20,000 IU.
[3] RSBM; or RSBM + HSBM (1:2); or HSBM; or HSBM + 0.55% inhibitor AA (thus supplying 0.11% inhibitor AA in the diet).

the consumed food were determined for each cage.

At the end of the trial the rats were killed, livers and kidneys were removed, weighed and tested for enzymatic activities.

The livers and kidneys from chicks and rats were prepared for various assays as follows: livers and kidneys were removed immediately after killing, weighed and put in cellulose nitrate tubes. The livers were homogenized in cold water at a ratio of 60 mg fresh weight/ml and the kidneys in a cold solution of 0.05 M $MnSO_4$ at a ratio of 20 mg fresh weight/ml for chicks and 30 mg/ml for rats. Each organ was homogenized for 20 seconds by Ultra Turrax at 20,000 rev/min.

Xanthine dehydrogenase and xanthine oxidase activities were determined by a modification of the procedure of Udenfriend (9) as follows: the homogenate was centrifuged for 20 minutes at 3300 *g* and 25 μliters of the supernatant were removed and inserted into a 12 mm × 100 mm test tube which was kept in a water bath at 30°. The reaction was started by the addition of 3 ml of 1 μM 2-amino 4-hydroxy pteridine [4] and 1 μM Na-EDTA (10), in 0.2 M phosphate buffer, pH 7.2 and the reaction mixture was mixed immediately. After 60 minutes the fluorescence intensity was measured in a Beckman DU spectrophotometer equipped with the 2980 fluorescence accessory, using Corning filter number 5860 (cut at 360 mμ) as primary and number 3389 (cut at 400 mμ) as secondary. Controls were measured immediately after addition of the pteridine solution to the homogenates. A solution of quinine sulphate (5 ppm quinine dissolved in 0.1 N H_2SO_4) served as a standard corresponding to 100% intensity. Reaction mixtures and controls were assayed in duplicates. One activity unit was defined as the increase in fluorescence intensity emitted by 3 ml reaction mixture after incubation during 60 minutes at pH 7.2, 30°, the intensity emitted by 1 ppm quinine in 0.1 N H_2SO_4 being taken as 100%.

Arginase activity was determined by the method of Cabello et al. (11) but with the following modifications for kidneys and livers. For kidneys: 0.5 ml of homogenate was diluted with 3.5 ml of 0.05 M $MnSO_4$. A sample of 50 μliters of the diluent was inserted into each of three 12 mm × 100 mm test tubes, one of which served as a control. The homogenates were activated in a 55° water bath according to Schimke (12) and transferred after 20 minutes to 37.5° water bath. The reaction was started by admixing the activated homogenate with 100 μliters of 0.1 M arginine·HCl in sodium glycine buffer, pH 10.14 (11). After exactly 10 minutes the reaction was terminated by addition of 3 ml of color reagent and the amount of liberated urea was determined (13). The appropriate controls were prepared by addition of the color reagent to the activated homogenates before the addition of the substrate. Solutions of 0.06, 0.12 and 0.18 μmole urea served as standards. For rat livers: Samples of 25 μliters of fresh homogenate were immediately mixed with 12.5 ml of 0.05 M $MnSO_4$ and the assay was then carried on as described for kidneys. One activity unit was defined as one micromole of urea released during 1 minute at 37.5°, pH 10.14, by the enzyme activated for 20 minutes at 55° in the presence of 0.05 M Mn.

The aminotransferases, alanine aminotransferase and aspartate aminotransferase, were determined according to Reitman and Frankel (14) on liver homogenates that had been diluted 10 times with water. One activity unit was defined as 1 mmole pyruvate released during 60 minutes at 37.5°.

[4] Kindly contributed by Lederle Laboratories Division, American Cyanamid Company, New York.

Twice distilled water was used throughout this work for the assays of enzymatic activities.

RESULTS

Trial 1. This trial corresponds to trial 1 in an earlier publication (8). Five out of 10 chicks were taken at random for enzyme determination. The experimental results are shown in table 3.

The extent of xanthine dehydrogenase activity corresponds with the growth indexes obtained for various soybean meal diets. The growth rates of the 5 chicks used for enzymatic determinations were similar to those of the whole groups. In the other enzymatic activities examined only the RSBM diets resulted in pronounced lower values. The differences obtained between other diets were not significant statistically. Diets of HSBM + CSBTI and HSBM + RSBM (2:1) indicated higher arginase activities which were found to be statistically significant in trial 1.

Trial 2. The results of this trial, which corresponds to trial 2 in an earlier publication (8), are summarized in table 4. The statistical factorial analyses of enzymatic activities of xanthine dehydrogenase and arginase were carried out by factorial analysis of variance (15) in 2 ways: (a) factorial analysis of all the groups fed at a 15% protein level, thus yielding five different protein sources in 2 levels of methionine (zero and 0.6%); and (b) factorial analysis of 4 groups fed different soybean meals: RSBM, RSBM + HSBM (1:2), HSBM and HSBM + AA (5.5 mg/g of HSBM), every group at 2 levels of protein and 2 levels of methionine, thus giving a 3-factorial (protein source, protein level, methionine) 4 × 2 × 2 experiment. Since no statistically significant interactions between factors were found, the differences between the three main effects, that is, protein source, addition of methionine and protein level, were calculated, and are shown in table 5.

In all cases the addition of methionine had no significant influence on the rate of enzymatic activities, whereas protein source and protein level resulted in statistically significant differences. As shown in table 5, the activities of liver xanthine dehydrogenase and kidney arginase were higher at the 25% protein level ($P < 0.01$). Xanthine dehydrogenase and arginase activities gave similar results when calculated per gram of tissue or per 100 g body weight. The activities of aspartate aminotransferase and alanine aminotransferase at the 25% protein level were higher too. The following 3 groups were found statistically different ($P < 0.05$) in liver xanthine dehydrogenase activity: 1) the group fed RSBM, 2) the group fed RSBM + HSBM (1:2) and 3) the group fed HSBM or HSBM supplemented by AA. Kidney arginase activity was found statistically lower only in animals fed diets with RSBM as the sole protein source and no significant differences were found between other diets. In two factorial statistical analyses of the enzymatic activities in lower protein level (15%) (that is, five protein sources and two methionine levels) xan-

TABLE 3

Effect of diets of raw soybean meal (RSBM), heated soybean meal (HSBM) and HSBM supplemented with trypsin inhibitors on enzymatic activities in livers and kidneys of chicks [1]

Protein source	Growth rate index [2]	In liver [3]			In kidney	In liver			In kidney
		XDH	GO-T	GP-T	Arginase	XDH	GO-T	GP-T	Arginase
		units/g tissue				*units/100 g body wt*			
RSBM	46	2.01 [a 4]	0.73 [a]	0.17 [a]	27.8 [a]	7.78 [a]	2.80 [a]	0.64 [a]	32.6 [a]
RSBM+HSBM (1:2)	76	3.44 [a]	0.81 [b]	0.18 [a]	61.7 [c]	13.24 [a]	3.08 [a]	0.68 [a]	78.2 [b]
HSBM	100	6.67 [b]	0.87 [b]	0.20 [a]	49.9 [b]	24.14 [b]	3.25 [a]	0.74 [a]	63.9 [ab]
HSBM+0.27% [5] AA	94	5.50 [b]	0.85 [b]	0.20 [a]	47.9 [b]	22.16 [b]	3.44 [a]	0.81 [a]	56.0 [ab]
HSBM+0.27% [5] CSBTI [6]	97	5.32 [b]	0.83 [b]	0.17 [a]	75.4 [c]	21.54 [b]	3.34 [a]	0.69 [a]	89.6 [b]

[1] Protein level 25%, 10 New Hampshire x Leghorn chicks/replicate, duration of trial 7 days.
[2] Calculated from table 4 (8) and expressed in percentage, taking the growth rate on HSBM diet as 100.
[3] XDH indicates xanthine dehydrogenase; GO-T, aspartate aminotransferase; and GP-T, alanine aminotransferase.
[4] All groups not designated with the same letter are statistically different ($P < 0.05$).
[5] Percentage in diet.
[6] CSBTI indicates crystalline soybean trypsin inhibitor (Kunitz units).

TABLE 4

Effect of addition of trypsin inhibitors at different levels of protein and methionine on enzymatic activities in livers and kidneys of chicks [1]

Diet no.	Protein level	Methionine added	Protein source [2]	Growth rate index [3]	In liver [4]			In kidney	In liver			In kidney
					XDH	GO-T [5]	GP-T [5]	Arginase	XDH	GO-T [5]	GP-T [5]	Arginase
	%	%			*units/g tissue*				*units/100 g body wt*			
1	15	0	RSBM	11	2.15	0.88	0.32	40.9	9.2	3.91	1.40	59.1
2			RSBM + HSBM (1:2)	28	6.44	1.01	0.33	74.4	27.2	4.50	1.47	95.7
3			HSBM	69	5.87	0.88	0.30	54.8	30.0	4.47	1.51	64.9
4			HSBM + 0.16% [6] AA [7]	67	6.57	1.04	0.30	59.0	31.6	4.99	1.43	83.2
5			HSBM + 0.48% [6] AA [8]	54	6.42	0.95	0.25	47.8	29.3	4.34	1.15	70.2
6	15	0.6	RSBM	31	2.20	0.95	0.26	31.4	10.1	4.38	1.21	37.3
7			RSBM + HSBM (1:2)	57	3.86	0.94	0.33	63.9	16.8	4.11	1.45	82.0
8			HSBM	79	7.36	1.08	0.30	56.7	34.7	5.06	1.42	68.1
9			HSBM + 0.16% [6] AA [7]	68	7.85	0.94	0.37	73.2	36.3	4.39	1.71	95.8
10			HSBM + 0.48% [6] AA [8]	64	5.56	0.95	0.35	57.4	25.4	4.34	1.58	76.1
11	25	0	RSBM	21	7.25	1.02	0.35	69.9	26.2	3.82	1.30	83.2
12			RSBM + HSBM (1:2)	58	9.82	1.02	0.36	103.1	47.9	4.74	1.68	144.1
13			HSBM	92	11.33	1.02	0.28	121.9	51.2	4.55	1.24	169.9
14			HSBM + 0.27% [6] AA [7]	80	13.07	1.08	0.37	100.1	59.8	4.99	1.69	158.0
15	25	0.6	RSBM	46	6.36	0.99	0.34	52.9	25.5	3.97	1.38	61.6
16			RSBM + HSBM (1:2)	71	9.75	1.06	0.34	83.8	47.5	5.13	1.63	116.4
17			HSBM	100	14.27	1.03	0.38	91.2	66.2	4.82	1.79	114.3
18			HSBM + 0.27% [6] AA [7]	84	12.39	0.99	0.38	85.2	62.3	4.49	1.72	118.7
19			HSBM + 0.27% [6] CSBTI	90	14.62	1.02	0.33	95.1	67.5	4.70	1.52	125.4

[1] Ten New Hampshire × Leghorn chicks/replicate, duration of trial 7 days; details of statistical analysis are given in text.
[2] RSBM indicates raw soybean meal; HSBM, heated soybean meal; CSBTI, crystalline Kunitz soybean trypsin inhibitor units; AA, trypsin and chymotrypsin inhibitor AA.
[3] Calculated from table 5 (8).
[4] XDH indicates xanthine dehydrogenase; GO-T, aspartate aminotransferase; and GP-T, alanine aminotransferase.
[5] Activities determined in 10 organs pooled together.
[6] Percentage in diet.
[7] 5.5 mg inhibitor/g HSBM.
[8] 16.5 mg inhibitor/g HSBM.

TABLE 5

Means of the main effect of addition of trypsin inhibitors at different levels of protein and methionine on enzymatic activities in livers and kidneys of chicks [1]

Main effect	Growth rate index [2]	In liver [3]			In kidney	In liver			In kidney
		XDH	GO-T [4]	GP-T [4]	Arginase	XDH	GO-T [4]	GP-T [4]	Arginase
		units/g tissue				*units/100 g body wt*			
Proetin level,									
15% [5]	74 [a 6]	5.29 [a]	0.97	0.31	56.8 [a]	24.5 [a]	4.48	1.45	73.3 [a]
25%	100 [b]	10.53 [b]	1.03	0.35	88.5 [b]	48.3 [b]	4.56	1.55	120.7 [b]
Protein source:									
RSBM	32 [a]	4.49 [a]	0.96	0.32	48.8 [a]	17.8 [a]	4.02	1.32	60.3 [a]
RSBM+HSBM (1:2)	63 [b]	7.47 [b]	1.01	0.34	81.3 [b]	34.9 [b]	4.62	1.56	109.6 [b]
HSBM	100 [d]	9.71 [c]	1.00	0.32	81.2 [b]	45.5 [c]	4.73	1.49	104.3 [b]
HSBM+AA [7]	88 [c]	9.97 [c]	1.01	0.35	79.4 [b]	47.5 [c]	4.72	1.64	113.9 [b]
Methionine,									
0.0% [5]	80 [a]	7.81 [a]	0.99	0.32	78.0 [a]	35.4 [a]	4.50	1.47	107.3 [a]
0.6%	100 [b]	8.01 [a]	1.00	0.34	67.3 [a]	37.4 [a]	4.54	1.54	86.8 [a]

[1] The HSBM + 0.48% AA and HSBM + 0.27% CSBTI treatments have been omitted in this presentation; RSBM indicates raw soybean meal; HSBM, heated soybean meal; CSBTI, crystalline Kunitz soybean trypsin inhibitor units; AA, trypsin and chymotrypsin inhibitor AA.
[2] Calculated from table 5 (8).
[3] XDH indicates xanthine dehydrogenase; GO-T, aspartate aminotransferase; and GP-T, alanine aminotransferase.
[4] Activities determined in 10 organs pooled together.
[5] Percentage in diet.
[6] All groups not designated by the same letter within the same main effect are statistically different ($P < 0.05$).
[7] 5.5 mg inhibitor/g HSBM.

thine dehydrogenase gave results similar to those of three factorial analyses (that is, four protein sources, two protein levels and two methionine levels), while in arginase no significant differences were found between different diets, probably because of great variability within each treatment.

The differences in enzymatic activities induced by HSBM and HSBM + CSBTI diets (table 4, diets no. 17, 19), were analyzed by *t* test and found not significant.

The activities of aspartate aminotransferase and alanine aminotransferase were generally slightly lower in chicks given RSBM diets than in others, as shown in table 4. Since the determinations were not performed individually no statistical analysis was carried out.

No differences were found between activity per gram of organ or per 100 g body weight as well as in the relative weight of kidneys and livers. An exception was the 25% protein RSBM diet which induced relatively lower liver weight, an effect which was not caused by the 15% protein RSBM diet.

The correlations between liver xanthine dehydrogenase and kidney arginase activities calculated per 100 g body weight and between these activities and growth rate (see table 5 in an earlier publication (8)) are shown in figure 1. The correlations were calculated for means of 19 treatments. Similar results were found in trials 1 and 3.

Trial 3. This trial was carried out with rats and corresponds to trial 3 in an earlier publication (8). Since no statistically significant interaction was found between sex and protein source and between litters and protein source the results presented in table 6 are means of all rats (males and females) given the same diet.

Data presented in tables 3–6 show that the influence of protein source on enzymatic activities of rats is similar to that for chicks, except for the activity of kidney arginase. The small statistical differences may be attributed to greater variability in trial 3.

DISCUSSION

The liver and kidney are among the body organs that are most sensitive to protein deficiency. Inadequate protein supplementation or administration of protein of low nutritive value will result in a greater rate of decrease in the protein content of liver and kidney than of proteins in other organs (16). Liver xanthine oxidase has

TABLE 6

Effect of diets of RSBM, HSBM and HSBM supplemented with trypsin inhibitors on enzymatic activities in livers and kidneys of rats [1]

Protein source	Growth rate index [2]	In liver [3]				In kidney	In liver			In kidney
		XO	GO-T	GP-T	Arginase	Arginase	XO	GO-T	GP-T	Arginase
		units/g tissue					*units/100 g body wt*			
RSBM	46 [a 4]	1.55 [a]	49.3 [a]	37.8 [a]	1290 [a]	9.20 [a]	8.0 [a]	246 [a]	188 [a]	6440 [a]	46.1 [a]
RSBM + HSBM (1:2)	78 [b]	2.84 [b]	51.3 [a]	40.3 [a]	1690 [b]	7.49 [a]	13.7 [ab]	250 [a]	197 [a]	8200 [b]	36.5 [a]
HSBM	100 [c]	3.47 [b]	51.9 [a]	37.4 [a]	1780 [b]	7.03 [a]	17.6 [b]	258 [a]	184 [a]	8900 [b]	35.0 [a]
HSBM + 0.27% [5] AA	85 [b]	3.20 [b]	51.0 [a]	39.8 [a]	1560 [b]	8.83 [a]	16.9 [b]	269 [a]	209 [a]	8210 [b]	46.5 [a]

[1] Protein level 10%, duration of trial 14 days; RSBM indicates raw soybean meal; HSBM, heated soybean meal.
[2] Calculated from table 6 (8) and expressed in percentage, taking the growth rate on HSBM diet as 100.
[3] XO indicates xanthine oxidase; GO-T, aspartate aminotransferase; and GP-T, alanine aminotransferase.
[4] All groups not designated with the same letter are statistically different ($P < 0.05$).
[5] Percentage in diet.

been reported to be highly influenced by protein source and level (17–19); protein diets with high efficiency induce high liver xanthine oxidase activities (20, 21). The enzyme possesses a wide substrate specificity and seems to be influenced by protein catabolic processes (22).

The influence of dietary proteins on the enzymatic activities involved in protein catabolism may originate in the availability of essential amino acids for protein synthesis, in the total quantity which reaches the liver and undergoes catabolic processes and in some factors in the diet that can inhibit protein synthesis, and especially the synthesis of enzymes. RSBM as a sole protein source causes a decrease in chick liver xanthine dehydrogenase and kidney arginase and rat liver xanthine oxidase and arginase activities when compared with HSBM. This finding is in agreement with the results of Shurpalekar et al. (23), Litwack et al. (24) and Borchers (25). The rat kidney arginase, however, was only slightly influenced by different diets. It should be pointed out that the arginase activity in the kidney of rats is negligible in comparison with this activity in liver, whereas in chick liver the arginase activity is extremely low (7). The heat treatment increases the availability of essential amino acids and releases perhaps also some other factors that enhance enzyme synthesis (26). According to the findings of Kwong and Barnes (27, 28) which have been supported by Borchers et al. (29), some trypsin-inhibiting factors influence also some metabolic processes and not only the intestinal absorption of amino acids.

It may perhaps be assumed that a combined induction mechanism is influencing the enzymes involved in protein catabolism: in one case the synthesis of catabolic enzymes is specifically inhibited and in the other, the total synthesis of organ protein is impaired. As a result more amino acids will arrive at the liver and undergo catabolic processes. These processes may induce the synthesis of catabolic enzymes (30). Such conditions exist for vitamin A deficiency in chicks for which a delay in protein synthesis was reported (31) and in which increases of liver xan-

thine dehydrogenase and kidney arginase activities were found.[5]

It appears that the ratio between the impairment of the total protein synthesis and the inhibition of synthesis of catabolic enzymes will vary to a great extent. The relative decrease in enzymatic activities caused by 15% protein diet was much higher than the relative decrease of growth rate (table 5). On the other hand, in chick diets supplemented with RSBM or with trypsin inhibitors (tables 3–5) the relative decrease in growth rate was higher than the relative decrease in enzymatic activities. In some cases the enzymatic activities of xanthine dehydrogenase and arginase were either not influenced at all or were even slightly higher than in HSBM control diet. These results indicate that

[5] Unpublished data.

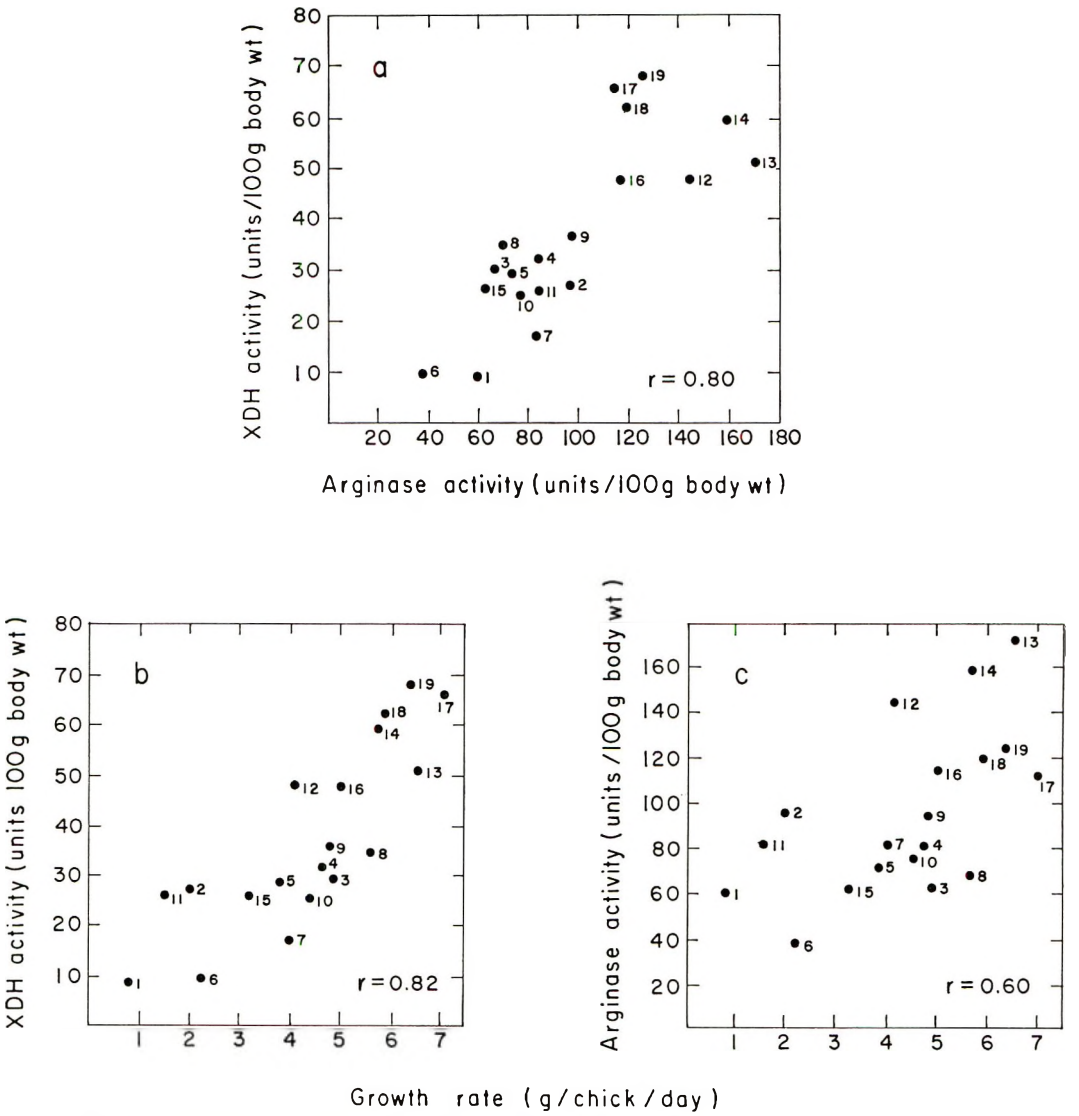

Fig. 1 Correlation between the means of (a) liver xanthine dehydrogenase and kidney arginase activities, (b) liver xanthine dehydrogenase activity and growth rate, and (c) kidney arginase activity and growth rate. The numbers correspond with the diet numbers in table 4.

the impairment caused by RSBM or trypsin inhibitors is of a different nature than the impairment caused by low protein diet. The CSBTI effect on kidney arginase activity (tables 3 and 4) resembles the increase in rat liver arginase resulting from diets supplemented with hydrocortisone, cortisone or iodinated casein, namely excess of favorable conditions for protein catabolism (32, 33).

The induction of liver aspartate and alanine aminotransferases by nutritive factors appears to be rather poor. At the 25% protein level there is some increase in activity as compared with that at the 15% protein level (table 5). The RSBM- and HSBM-supplemented diets appear to affect these enzymatic activities only very slightly.

Our findings that methionine supplementation does not increase enzymatic activities are in agreement with the results of de Monterice et al. (34) and do not support the findings of Shurpalekar et al. (23) and Williams et al. (35) about a considerable increase in xanthine oxidase activity in rats.

The correlation between liver xanthine dehydrogenase and arginase activities (fig. 1) indicates that these 2 enzymes are similarly influenced by protein metabolism processes in the body. The close relationship between kidney arginase and liver xanthine dehydrogenase activities, as well as the good correlation between the activities of these enzymes and the growth rate, may suggest the use of these enzymatic activities as criteria for growth rate estimation, and for studying influences of the diet on protein catabolism.

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Significance of the Endogenous Nitrogen Excretion in Protein Evaluation Studies with Rats [1]

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ABSTRACT Four experiments with young male rats were carried out to determine whether any particular urinary N constituents varied closely with the quantity and quality of dietary protein and to compare the "endogenous" N loss determined from the excretion of rats fed an N-free diet with that calculated from regression equations relating urinary N excretion to dietary protein intake. The dietary protein sources, which included casein and isolated soy with and without methionine supplementation, were fed at zero, 2, 4, 6, and 10% levels. The total urinary nitrogen excretion with a protein-free diet was much higher than the calculated value from the regression relating protein intake to total urinary nitrogen excretion extrapolated to zero N intake. The extrapolated values for total nitrogen excretion at zero N intake for the various experiments and different levels of dietary protein varied considerably, as did the observed nitrogen excretion values of the rats fed the protein-free diet. In contrast with these variable results, the non-urea nitrogen values, calculated from the regression equations relating total urinary N and urinary urea nitrogen to protein intake, were remarkably similar at about 50 mg/m². Biological values, calculated by the Thomas-Mitchell method, differed greatly from values determined by the direct carcass nitrogen retention method. When the Thomas-Mitchell equation was modified by substituting the calculated non-urea nitrogen value for the "endogenous" nitrogen there was good agreement of the biological values thus calculated with those obtained by the carcass retention method. The significance of the relation of these findings to the concept of "endogenous" nitrogen metabolism is discussed.

Renewed interest has been stimulated in the relationship between quantity and quality of dietary protein and the nitrogen (N) excretion products in the urine. Njaa recently (1) suggested that the measurement of the "endogenous" N excretion by the original Thomas-Mitchell (2) method, that is, the N excreted by animals fed an N-free diet, may introduce considerable and possibly irrelevant variation into the estimation of biological value (BV). Njaa showed that the urinary N excreted by animals fed an N-free diet did not remain constant after a period of adaptation, but varied with time and in response to alterations in diet and physiological status of the animal. Similar observations had been reported earlier by French et al. (3).

Holmes (4) has also severely criticized the accuracy and meaningfulness of the use of the "endogenous" N excretion in estimating recommended protein allowances. Further doubt concerning the constancy and applicability to BV determinations of the urinary N of animals fed a N-free diet was cast by the results of comparative studies by Henry (5) and by Chalupa and Fisher (6). These workers found generally higher net protein utilization (NPU) values for proteins evaluated by the Thomas-Mitchell balance sheet method as compared with the Miller-Bender (7) carcass N retention method.

The present studies were undertaken (a) to determine whether any particular urinary N constituents varied closely with the quantity and quality of dietary protein, and (b) to compare the "endogenous" N loss determined from the excretion of rats on a N-free diet with that calculated from regression equations relating urinary N excretion to dietary protein intake.

METHODS AND MATERIALS

The investigation was carried out in 4 experiments which differed primarily in the protein and amino acid content of the

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experimental diets. Casein served as the test protein in experiments 1 and 2, and isolated soy-protein was used in experiments 3 and 4. DL-Methionine was an additional dietary variable in experiments 2 and 4. Table 1 shows the composition of experimental diets.

Twenty-four male albino rats of the Charles River strain, weighing approximately 285 g each, were used in each experiment. The animals were divided on the basis of similar body weight into 4 groups of five and one group of four, the latter serving as the negative control group, that is, the group which was fed a protein-free diet for the duration of the experiment. The other 4 groups of rats were fed 2, 4, 6, and 10% levels of dietary protein, respectively. Daily, all animals within each experiment were given the same amount of food, equivalent to the smallest quantity consumed by any animal during a pre-experimental feeding period. Food intakes, and therefore N intakes, were similar for the rats in experiments 1, 2, and 3. The amount of food consumed by animals in experiment 4 was substantially greater than that for the other 3 experiments. The rats were trained to eat all their food in two 2-hour intervals 12 hours apart. This training was instituted to minimize food spillage and contamination of the urine and feces by the food.

In accordance with the Thomas-Mitchell procedure, the animals were first fed the protein-free ration for 10 days (period 1) followed by the feeding of the test ration for 8 days (period 2). Urine and feces were collected the last 4 days of each period. The feces from each animal were pooled for each collection period and analyzed for total N by a macro-Kjeldahl procedure (7). Urine was collected for each 24-hour period, with metaphosphoric acid used as a preservative. The urine was then diluted to a volume of 25 ml with distilled water and analyzed for total N by a semi-micro-Kjeldahl technique (8), for urea (9), for creatinine [2] (10), and for uric acid (11).[3] All urinary analyses were completed on the day of collection.

Eighteen hours after the final feeding the animals were killed with chloroform. The carcasses were dried to constant weight and ground in a meat grinder; 0.5-g portions of the ground carcass were analyzed for total N by the macro-Kjeldahl method.

TABLE 1

Composition of experimental diets

	%
Cornstarch	28.0
Corn dextrin	5.0
Fiber	3.0
Corn oil	5.0
Choline chloride (70%)	0.3
Vitamin mix (10)	0.2
Mineral mix (10)	4.9
Casein (exps. 1 and 2)	variable
Isolated soy-protein [1] (exps. 3 and 4) [2]	variable
DL-Methionine (exps. 2 and 4)	0.2
Sucrose	to make 100

[1] Nutritional Biochemicals Corporation, Cleveland.
[2] Assay Protein C-1, Skidmore Enterprises, Cincinnati.

RESULTS AND DISCUSSION

Table 2 shows the N balance results for all experiments, expressed as mg N/m^2 body surface. Body surface was calculated by the equation of Lee (12): $A_{cm}^2 = 12.54\ W_g^{0.60}$. With one exception, negative N balances were observed at the 4% and 2% levels of protein, and apparently reflect an inadequate dietary supply of total N. In experiment 3 (soy-protein) only the 6% protein group was in positive N balance; all other groups were in negative N balance. The anomalous data with soy-protein are not susceptible to convincing explanation; the observations may be related to the relative magnitude of the sulfur amino acid deficiency at different levels of protein.

Table 3 shows the average body composition of the animals in all experiments, pooled by treatment group. It should be noted that animals that were in positive N balance (table 2) during the test feeding period (period 2) had more body N than the animals fed the diets resulting in negative N balance. Animals fed the unsupplemented soy-protein had the lowest body N of all the protein-fed groups. All these animals also lost weight, while in other experiments weight loss was char-

[2] Results of these analyses are not included in this text but have been deposited with the American Documentation Institute, Library of Congress.
[3] See footnote 2.

TABLE 2

Nitrogen balance data of rats fed diets containing various levels of casein or isolated soy-protein with and without 0.2% DL-methionine supplementation [1]

Diet	N intake		N excretion				N balance
			Urine		Feces		
	Period 1	Period 2	Period 1	Period 2	Period 1	Period 2	
	mg/m²	*mg/m²*	*mg/m²*	*mg/m²*	*mg/m²*	*mg/m²*	*mg/m²*
			Experiment 1				
10% casein	—	5268	1145 ± 58 [2]	2429 ± 75 [2]	427	602	2237
6% casein	—	3150	1170 ± 64	1595 ± 58	456	604	950
4% casein	—	2294	1142 ± 62	1244 ± 43	455	506	544
2% casein	—	1114	1196 ± 63	928 ± 50	484	477	− 391
Protein-free	—	—	1105 ± 75	657 ± 21	549	422	− 1098
			Experiment 2				
10% casein + methionine	—	5107	1386 ± 12	1943 ± 12	611	860	2484
6% casein + methionine	—	3241	1058 ± 62	1382 ± 63	588	696	1162
4% casein + methionine	—	2233	1033 ± 32	1122 ± 61	563	653	458
2% casein + methionine	—	1246	1128 ± 54	1067 ± 71	571	557	− 378
Protein-free	—	—	934 ± 88	763 ± 31	517	441	− 1204
			Experiment 3				
10% soy	—	5243	1497 ± 16	4604 ± 28	444	705	− 366
6% soy	—	3125	1243 ± 69	2347 ± 91	518	559	289
4% soy	—	2250	1334 ± 52	1987 ± 85	579	660	− 398
2% soy	—	1306	1313 ± 60	1529 ± 57	493	493	− 716
Protein-free	—	—	1146 ± 58	932 ± 40	538	431	− 1364
			Experiment 4				
10% soy + methionine	—	8180	1775 ± 59	3030 ± 10	594	938	4211
6% soy + methionine	—	5086	1644 ± 48	2053 ± 69	607	885	2148
4% soy + methionine	—	3463	1760 ± 59	1777 ± 79	573	852	834
2% soy + methionine	—	2084	1744 ± 49	1276 ± 55	533	670	48
Protein-free	—	—	1697 ± 81	1153 ± 33	648	744	− 1898

[1] Period 1, fed protein-free diet for 10 days; period 2, fed diets in column 1 for 8 days.
[2] Mean ± SE.

acteristic only of the 2% protein and the protein-free groups.

Table 4 shows the urinary urea N excretion of the animals in all experiments. Analysis of variance showed that both the urea N and the total urinary N were significantly and similarly affected by the quantity of protein fed and by the addition of methionine. Regression analysis (table 5) of the excretion data from period 2 in each experiment (tables 2 and 4) showed that the slope of the line relating N intake to total urinary N was the same as the slope of the line relating N intake to urinary urea N. Goodness of fit test showed all lines to be linear at $P < 0.01$. The parallelism between the 2 lines suggested that the difference between the absolute values of Y at $X = O$, or the non-urea N, may well be a measure of constant biological processes heretofore defined as the "endogenous" N metabolism.

In the Thomas-Mitchell method of determining the BV of protein, the N excretion of animals fed an N-free diet after a period of adaptation is used as a measure of "endogenous" N. This value for all groups of animals fed the N-free diet during period 1 in the present study ranged from 934 to 1775 mg/m² (table 2). By contrast, the values for the non-urea N at zero N intake were approximately the same at 500 mg/m² in experiments 1, 2 and 4.

Table 6 shows BV's calculated for each of the test diets by the Thomas-Mitchell equation with (a) the total urinary N on a protein-free diet as U′ and (b) the extrapolated non-urea N excretion at zero N

TABLE 3

Body composition of rats fed diets containing various amounts of casein or isolated soy protein with and without 0.2% DL-methionine supplementation

Test diet	Animals	Average carcass weight		Water	N	Total body N [1]
		Wet	Dry			
		g	*g*	*%*	*% dry weight*	*g*
		Experiment 1				
10% casein	5	297	112	62.2	9.15	10.25
6% casein	5	294	102	65.1	9.58	9.77
4% casein	5	309	106	65.7	9.02	9.56
2% casein	5	297	108	63.6	8.77	9.48
Protein-free	4	283	106	62.5	8.86	9.39
		Experiment 2				
10% casein + methionine	5	275	95	65.2	10.22	9.71
6% casein + methionine	5	269	94	65.0	9.86	9.26
4% casein + methionine	5	266	89	66.0	9.96	8.86
2% casein + methionine	5	264	96	63.4	9.07	8.80
Protein-free	4	255	90	64.8	9.69	8.73
		Experiment 3				
10% soy	5	215	67	68.7	11.17	7.48
6% soy	4	226	75	66.9	10.18	7.64
4% soy	5	217	71	67.3	10.42	7.40
2% soy	5	207	68	66.9	10.25	6.97
Protein-free	4	210	72	65.6	9.62	6.93
		Experiment 4				
10% soy + methionine	5	276	94	66.0	9.36	8.80
6% soy + methionine	5	259	87	66.6	9.20	7.96
4% soy + methionine	5	255	101	60.3	7.80	7.91
2% soy + methionine	5	245	99	59.7	7.70	7.62
Protein-free	4	236	88	62.9	8.23	7.29

[1] % N × dry weight.

TABLE 4

Urea excretion of rats fed diets containing various levels of casein or isolated soy-protein with and without 0.2% DL-methionine supplementation [1]

Diet	N intake		Urea N excretion	
	Period 1	Period 2	Period 1	Period 2
	mg/m²	*mg/m²*	*mg/m²*	*mg/m²*
	Experiment 1			
10% casein	—	5268	870 ± 127 [2]	2109 ± 86
6% casein	—	3150	1030 ± 102	1108 ± 103
4% casein	—	2294	920 ± 113	861 ± 65
2% casein	—	1114	816 ± 119	471 ± 58
Protein-free	—	—	867 ± 350	318 ± 33
	Experiment 2			
10% casein + methionine	—	5107	1108 ± 92	1059 ± 66
6% casein + methionine	—	3241	803 ± 54	814 ± 54
4% casein + methionine	—	2233	772 ± 52	474 ± 50
2% casein + methionine	—	1246	754 ± 62	434 ± 49
Protein-free	—	—	609 ± 42	269 ± 39
	Experiment 3			
10% soy	—	5243	1253 ± 79	3908 ± 278
6% soy	—	3195	925 ± 76	1645 ± 64
4% soy	—	2250	1193 ± 91	1409 ± 42
2% soy	—	1306	1045 ± 78	1164 ± 52
Protein-free	—	—	849 ± 73	450 ± 28
	Experiment 4			
10% soy + methionine	—	8180	1055 ± 52	1885 ± 81
6% soy + methionine	—	5086	980 ± 60	1167 ± 47
4% soy + methionine	—	3463	1112 ± 79	952 ± 59
2% soy + Methionine	—	2084	1056 ± 54	654 ± 36
Protein-free	—	—	940 ± 86	469 ± 45

[1] Period 1, fed protein-free diet for 10 days; period 2, fed diets in column 1 for 8 days.
[2] Mean ± SE.

TABLE 5

Regression analysis of urea N and total urinary N vs. N intake

Excretion product	Equation of line [1]	Correlation coefficient r	Calculated non-urea N at X = 0
	Casein (exp. 1)		
Urea N	41.5 X + (−53.7) = Y	0.670	500.3
Total N	38.4 X + 446.6 = Y		
	Casein + methionine (exp. 2)		
Urea N	17.7 X + 182.5 = Y	0.724	494.9
Total N	24.2 X + 677.4 = Y	0.704	
	Soy (exp. 3)		
Urea N	77.2 X + (−189.6) = Y	0.844	340.6
Total N	85.7 X + 151.0 = Y	0.859	
	Soy + methionine (exp. 4)		
Urea N	20.2 X + 271.2 = Y	0.868	511.5
Total N	28.3 X + 782.7 = Y	0.877	

[1] X = N intake (2–10% of diet), period 2, table 2; Y = N excretion (urea N or total N), period 2, tables 2 and 4.

intake used for U′; these values were compared with BV's calculated from the carcass N data according to the Miller-Bender method. The BV's calculated by method (a) varied inversely with level of dietary protein, in agreement with earlier reports (13). Using the extrapolated non-urea N for these calculations (method b) the BV's were remarkably similar for experiments 1, 2 and 4, and essentially independent of protein intake. Such independence of BV from N intake was recently demonstrated by Hegsted (14) in growing rats when BV was expressed in NPU equivalents. Nitrogen balance indexes (NBI's) calculated according to a modified Allison (15) method were essentially independent of protein intake for experiments 1 and 2, and lower than the BV's calculated by method (a). They were, however, further from the BV's of the Miller-Bender method than those derived from method (b), as might be predicted because of their dependence on total urinary N excretion, and in 3 of the 4 experiments more variable than method (b).

GENERAL COMMENTS

The so-called "endogenous N" has been measured in a variety of ways, all using the total N excreted by animals fed a protein-free diet in: a) a protein-free period for each group, preceding the protein-feeding period as in the original Thomas-Mitchell method, (method a, table 6): b) the average of 2 protein-free periods, one before and one after the test period, as in Allison's NBI method; and c) period 2 of a separate group of animals continuously fed a protein-free diet; the excretion value for that period is used as "endogenous N" for groups fed the test proteins after an initial period of protein-free period (modified NBI method, table 6). Each of these procedures is open to the criticism that total urinary N of animals fed a protein-free diet is greater than "endogenous N" as defined by Holmes (4) as the sum of the necessary by-products of cell activities under normal circumstances; rather, it reflects the breakdown of body protein to meet the needs of the protein restriction. This distinction was repeatedly demonstrated by Allison and others, who found that in some cases, notably with methionine and with egg protein, "feeding N conserves body N"; and total N excretion falls.

The evaluation of a protein requires measurement of the effect of the protein per se; thus the extent of the N metabolism which is ongoing and independent of the kind and amount of protein fed must be accounted for. The calculation of the extrapolated non-urea urinary N at zero N intakes gives, as Frost suggested in his discussion (15) "good representative values which will fit a strict empirical definition" of endogenous N. Use of such an

TABLE 6

Comparison of the biological values of casein and soy protein with and without DL-*methionine calculated by two modifications of the Thomas-Mitchell method and the Miller-Bender carcass N retention method and the nitrogen balance index*

Test diet	Thomas-Mitchell method [1]		NBI [2]	Miller Bender method [3]
	a	b		
	Experiment 1			
10% casein	74.8	62.2	0.656	55.5
6% casein	85.8	63.4	0.699	
4% casein	95.5	66.8	0.743	
2% casein	124.1	61.8	0.668	
	Experiment 2			
10% casein + methionine	88.9	71.4	0.787	66.2
6% casein + methionine	89.7	72.2	0.792	
4% casein + methionine	95.9	71.0	0.822	
2% casein + methionine	104.9	54.5	0.731	
	Experiment 3			
10% soy	36.9	14.4	0.200	44.2
6% soy	65.0	35.0	0.552	
4% soy	69.9	24.1	0.478	
2% soy	83.5	14.4	0.521	
	Experiment 4			
10% soy + methionine	84.0	67.8	0.765	67.8
6% soy + methionine	91.5	67.9	0.818	
4% soy + methionine	99.4	60.2	0.814	
2% soy + methionine	125.2	58.8	0.902	

[1] $BV = 100 \times \frac{I - (U - U')}{I}$

When I = absorbed N of animals fed a test protein ration
U = N excreted in the urine of animals fed a test protein ration
U′ = N excreted in the urine of animals fed a protein-free diet
Modification a U′ = total urinary N on protein-free diet (values from period 1, table 2)
b U′ = extrapolated non-urea urinary N at zero N intake.

[2] NBI (nitrogen balance index) $= \frac{NB_I - NB_0}{I}$

When I = absorbed N of animals fed a test protein ration
NB_I = N balance of animals fed a test protein ration
NB_0 = N balance of animals fed a protein-free ration
(Protein-free groups, period 2, table 2).

[3] $BV = \frac{Bf - BK + IK}{If} \times 100$

When Bf = carcass n of animals fed test protein diet
If = absorbed N of animals fed test protein diet
Ik = absorbed n of animals fed protein-free diet.

empircial definition (method b) gives values for proteins that are closer to the ultimate measure of nitrogen retention, carcass analysis, than those obtained by the original Thomas-Mitchell method or by the NBI.

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Milk Levels of Selenium and Vitamin E Related to Nutritional Muscular Dystrophy in the Suckling Lamb [1]

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ABSTRACT Se, as Na_2SeO_3, was added to ewes' rations containing raw and autoclaved kidney beans to compare bean-heating effect on utilization of Se in preventing nutritional muscular dystrophy (NMD) in suckling lambs. Ration effects on digestibility, milk production, milk composition and daily lamb intake of Se and vitamin E were examined. Observations were also made on distribution of ^{75}Se in lamb tissues in relation to treatment effects. Supplemental Se and heat treatment of beans reduced the incidence of NMD. Autoclaving increased the apparent digestion of bean Se, as well as increasing milk production and daily milk yield of Se and vitamin E. Addition of Se appeared to reduce milk fat but no other treatment effects on major chemical components were noted. ^{75}Se consumed in the ewes' milk was distributed in lamb tissue in approximately equal proportion due to treatments, with the possible exception of the pancreas.

The cause of nutritional muscular dystrophy (NMD) frequently associated with feeding a basal ration of locally grown hay and cull kidney beans (*Phaseolus vulgaris*) has been the subject of numerous investigations at this laboratory (1–3). Attempted preventive treatments have included addition of vitamin E, Se, antioxidants and sulfur-containing amino acids to the rations of the ewes or their lambs, and autoclaving of the beans and hay. A combination of vitamin E and Se given to the ewes resulted in complete protection for the lambs, and yet each fed separately was only partially effective. Complete protection was also realized when both the hay and beans were autoclaved. This led Hogue et al. (2) to propose that either a heat-labile antagonist of some type was present in the beans and hay, acting in some way to enhance muscular dystrophy in the lamb, or that heat treatment simply enabled the animal to better utilize the vitamin E and Se in the ration. Hintz and Hogue (4) reported an apparent antivitamin E effect when kidney beans were included in the diet of chicks. They suggested that this antagonist was likely the causative agent in NMD of the lamb.

Milk is normally the sole source of nutrients consumed by lambs when they become dystrophic but little attention has been given to the total daily milk consumption or its qualitative characteristics which might predispose the lamb to NMD. Any possible antimetabolite to Se or vitamin E, or to both, contained in beans must be transmitted to the lamb via the milk in order to interfere with normal metabolism.

Considering the above factors the purposes of this experiment were to: 1) examine the antidystrophogenic value of supplemental inorganic Se when added to a ration containing autoclaved and raw beans; 2) measure digestibility effects on milk production and milk composition of ewes consuming raw and autoclaved beans; 3) trace the incorporation of labeled Se into milk and subsequent distribution into lamb tistue, to detect treatment effects on distribution of Se; and 4) determine the presence or absence of an antagonist to Se or vitamin E, or to both, in beans.

EXPERIMENTAL

Fifty-two western whiteface ewes, with their lambs, were assigned at random to 4 treatment groups. Five ewes in each treatment group were suckling twin lambs, and the remainder single lambs. Treat-

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ments included: 1) basal ration of trefoil grass mixed hay and cull kidney beans (*Phaseolus vulgaris*); 2) basal ration plus 2.25 mg Se/ewe per day; 3) basal hay with autoclaved kidney beans; and 4) basal hay, autoclaved beans and 2.25 mg Se/ewe per day. Sodium selenite was used as a source of Se and the level added approximates 1.0 ppm of the total diet. The mixed hay and beans were known to be dystrophogenic from previous studies with these feeds, grown in specific fields in central New York State.

The basal diet was fed to the ewes approximately one month before parturition of the first ewe. All ewes were group-fed an average of 900 g of kidney beans and 1360 g of hay daily. Forty-five grams of dextrose were offered to each ewe/day as a carrier for the sodium selenite which was added to the dextrose of treatment groups 2 and 4 at a level to provide 2.25 mg of selenium/ewe per day. The lambs did not have access to supplemental feed and accordingly consumed a small amount of the ewes' rations.

The beans were autoclaved at 118° for 30 minutes in a large soil sterilizer. They were fed within a week after autoclaving to prevent spoilage.

Lambs were weighed weekly and at the termination age of 60 days. Blood samples were obtained by jugular puncture when the lambs were 2, 4, 6 and 8 weeks of age. Blood serum glutamic oxalacetic transaminase (SGO-T) levels were determined on all blood samples by a colorimetric procedure.[2] SCO-T levels in excess of 200 Sigma-Frankel units were considered to indicate muscular dystrophy (5). Autopsies verified this conclusion. The lambs were forced to run each day in order that clinical signs of stiffness could be observed.

Milk production and milk composition of the ewes was determined during the third and sixth weeks of lactation. Milk production was determined by separating the ewes from their lambs and then emptying the udder by hand milking following intrajugular injections of 5 USP units of a purified oxytocic principle[3] in 5 ml of saline at the beginning and end of a 2.5- to 3-hour interval. The milk produced during this time-interval was used to assess the quantity of milk consumed by the lamb inasmuch as this is the normal suckling interval of lambs of this age (6). Milk dry matter was determined by freeze-drying, fat content by the Babcock test and protein by multiplying the nitrogen content (Kjeldahl procedure) by 6.38. Se content of the milk and other samples was measured by a fluorometric method (7). Milk sulfur levels were determined by X-ray fluorescence analysis.[4] A modified method of Quaife and Harris (8) was used in tocopherol determinations. Amino acid content of the milk was obtained by use of an amino acid analyzer.

A digestion study was conducted with 3 nonlactating ewes to compare the digestibility of autoclaved versus raw beans. The effect of autoclaving on apparent intestinal absorption of Se was observed concomitant with the digestion study.

One hundred microcuries of ^{75}Se, as selenite, were given orally by capsule to each of 2 ewes suckling lambs from treatment 1, and 2 ewes from treatment 3. After a time lapse of 6 days, counts were made on the ^{75}Se in the milk and selected tissues of the lamb bodies.

RESULTS AND DISCUSSION

Incidence of NMD. Supplementing the ewes' basal ration with 2.25 mg Se/day reduced the incidence and severity of NMD in their lambs (table 1). This beneficial effect is understandable in view of the very limited quantity of Se and tocopherol in the basal ration (table 2). Autoclaving the beans likewise reduced the incidence and severity of NMD but the most effective preventive combination was that of cooked beans plus supplemental selenium. The only lambs with elevated SGO-T values in this treatment group were 1 pair of twins. The 2 lambs without elevated SGO-T values in the basal treatment group were single lambs. Fifty percent of the single lambs were affected by NMD on all treatments and 60% of the twin lambs.

Three lambs with elevated SGO-T values were autopsied. One of the three showed a

[2] Sigma Technical Bulletin 505, Sigma Chemical Company, St. Louis.
[3] P.O.P., Armour Pharmaceutical Company, Kankakee, Illinois.
[4] Unpublished method, V. A. Lazar, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, New York.

TABLE 1

Effects of supplemental Se and autoclaved beans on lamb weights, incidence and severity of nutritional muscular dystrophy (NMD)

		No. lambs	Avg lamb wt at 60 days		Clinically affected lambs [1]	Lambs with elevated SGO-T				
			Singles	Twins		Wk 2	Wk 4	Wk 6	Wk 8	Total over 9-wk period
			kg	*kg*	%	%	%	%	%	%
1	Basal ration (hay + raw beans)	18	20.8	13.2	22.2	18.8	55.6	72.2	87.5	88.9
2	Basal ration + 2.25 mg Se/day as Na_2SeO_3	18	19.4	15.6	0	0	5.6	27.8	38.8	50.0
3	Basal hay + autoclaved beans	17	21.7	15.3	5.9	0	0	30.0	66.6	66.6
4	Basal hay + autoclaved beans + 2.25 mg Se/day as Na_2SeO_3	19	22.2	16.3	0	0	10.5	10.5	10.5	10.5

[1] Clinically affected lambs were determined daily by visual appraisal.

TABLE 2

Selenium content of ration

	Se
	ppm
Mixed hay	0.02
Kidney beans	0.03

marginal SGO-T value indicative of NMD. The semimembranosus and semitendinosus muscles of all three were pale and displayed varying degrees of necrotic change upon cross section examination. No necrotic changes in heart muscles were observed upon gross examination.

Milk production and composition. Treatment effects on milk production and milk composition are summarized in table 3. Ewes consuming autoclaved beans produced significantly ($P < 0.05$) more milk than those consuming the same quantity of raw beans, resulting in heavier lambs at 60 days of age (table 1). Differences were established as early as the third week of lactation. Ewes with twin lambs produced an average of 17% more milk than those with single lambs during the third week of lactation but production values were not different after 6 weeks of lactation. The increase in production due to autoclaving the beans could not be attributed to greater digestibility of the major chemical components of the ration since no differences were observed in digestion coefficients or digestible energy values.

The only treatment effect on major milk nutrients (table 3) was an apparent reduction in the percentage of milk fat due to the addition of supplemental Se to the ewes' rations. Although milk protein percentages were not affected by treatment factors, daily protein yields were increased by autoclaving the beans (table 3). Amino acid composition of the milk did not differ between treatments (table 4). Milk from ewes whose lambs exhibited elevated SGO-T values was equivalent in amino acid composition to milk producing no NMD (table 4). The comparative amounts of selenomethionine versus methionine and selenocystine versus cystine were not examined. Milk sulfur content was approximately equal between treatments (table 5). A milk sulfur level above the group average was observed in milk from ewes fed the basal ration whose lambs exhibited SGO-T values indicative of NMD (table 5, footnote). Jones and Godwin (9) reported that when inorganic ^{75}Se was injected intravenously into a ewe, 73% of that appearing in the milk was in the protein fraction. Such incorporation of Se into the amino acids of the milk in this study appears likely. Results of this study, together with those of Hintz and Hogue (3), discount the role of the sulfur-containing amino acids per se, as opposed to Se and vitamin E, in the prevention of NMD in the suckling lamb. However, substitution of Se for S in these amino acids has been pro-

TABLE 3

Effect of autoclaving beans and supplemental Se on milk production and composition

	Treatment no.							
	1		2		3		4	
	No. lambs suckled		No. lambs suckled		No. lambs suckled		No. lambs suckled	
	1	2	1	2	1	2	1	2
Avg milk production, 3rd week of lactation g/24 hr	1533	1582	1518	1861	1577	1940	1782	2020
Avg milk production, 6th week of lactation, g/24 hr	1111	1029	1189	1240	1431	1747	1483	1558
Dry matter, % [1]	18.99		18.65		19.66		17.91	
Fat, %	8.0		7.7		9.0		7.3	
Caloric value, kcal/g	1139		1111		1195		1050	
Protein, %	4.63		4.72		4.64		4.38	
Total protein production, g/24 hr	49.97		57.11		68.67		66.11	

[1] Composition values represent analyses of milk composited from all ewes in respective treatment groups during sixth week of lactation.

TABLE 4

Milk amino acid composition as affected by autoclaving beans and supplemental Se [1]

Amino acid	Treatment no.				Elevated SGO-T [2]
	1	2	3	4	
	% total amino acids determined				
Aspartic	8.7	9.1	9.5	9.4	9.5
Threonine	5.3	5.9	5.7	5.8	5.9
Serine	6.9	8.1	7.6	7.8	8.2
Glutamic	19.0	19.7	20.1	20.2	20.3
Glycine	3.9	4.2	4.2	4.2	3.9
Alanine	5.9	6.4	6.6	6.3	6.8
Valine	6.6	6.6	6.5	6.7	6.6
Methionine	2.0	2.1	2.1	2.1	2.1
Isoleucine	6.2	5.2	5.5	5.2	5.3
Leucine	11.1	10.9	10.6	11.1	10.8
Tyrosine	3.5	3.2	3.1	3.4	3.2
Phenylalanine	4.5	4.2	4.0	4.3	4.2
Lysine	10.1	8.6	8.9	8.0	7.8
Histidine	3.0	2.8	2.6	2.7	2.7
Arginine	3.5	2.8	3.0	2.8	2.8

[1] The results were obtained with an amino acid analyzer equipped with one colorimeter set at 570 mμ. As a result, the relative error on the proline reading was large and this amino acid is not listed. During acid hydrolysis the cystine was converted partly to cysteic acid and an estimation of cystine was not attempted. The amount of cysteic acid was the same in all 5 milk protein samples.
[2] Milk from ewes whose lambs had elevated SGO-T values.

posed (10) as the biological means through which Se acts in preventing NMD.

Milk Se and vitamin E. Autoclaving the beans and supplementing the ration with inorganic Se increased the daily production of milk Se, although in some instances concentrations were the same on a dry matter basis (table 5). The conversion of supplemental Se to milk Se was likewise enhanced by autoclaving the beans. Milk Se concentrations declined from the third to sixth weeks of lactation on treatments 1 and 3, whereas they remained relatively constant on the supplemental Se rations, perhaps indicating a depletion in the ewes' body stores with the former rations. Con-

centration decline coupled with milk yield declines reduced daily Se production to 56 and 61%, respectively, of values during the third week. The time of decline corresponded with increased incidences of NMD.

One important effect of autoclaving the beans and supplementing the raw bean ration with Se was to increase the daily yield of tocopherol (table 5). Added Se elevated daily tocopherol production by one-third, possibly due to a sparing or synergistic effect in the metabolism of the ewe. Autoclaving elicited a 180% advantage over the basal ration. These results show an additive effect between vitamin E and Se in the prevention of NMD in the lamb. Calvert and co-workers (11) and Maplesden and Loosli (12) have shown that vitamin E is required in addition to Se in prevention of NMD in the chick and calf, respectively. The same appears to be true of the lamb.

Absorption and utilization of Se and vitamin E. Results of the digestion-balance study showed that autoclaving beans increased the apparent availability of Se and vitamin E (table 6). Ewes consuming raw beans showed a negative Se balance of 5.5 μg/day. How much of the Se was absorbed and then excreted back into the gut is not known. Peterson and Spedding (13) have shown that the major path-

TABLE 5

Quantities of Se, tocopherol and sulfur in milk as affected by autoclaving beans and supplemental Se

	Treatment no.			
	1	2	3	4
Se concentration, 3rd week of lactation (dry matter basis), ppm [1]	0.078	0.20 [2]	0.081	0.33
Avg production milk Se/ewe/24 hr, 3rd week of lactation, μg	22.0	64.0	28.7	107.8
Se concentration, 6th week of lactation (dry matter basis), ppm [1]	0.06	0.21	0.06	0.32
Avg production milk Se/ewe/24 hr, 6th week of lactation, μg	12.28	47.43	17.46	86.29
Tocopherol, concentration, 6th week of lactation (dry matter basis), ppm [1]	4.12	4.98	5.35	5.59
Avg production of milk tocopherol/ewe/24 hr, mg	0.84	1.12	1.53	1.51
Sulfur levels, 3rd week of lactation (dry matter basis), %	0.23 [3]	0.24	0.23	—

[1] Determined on composite samples from each treatment group.
[2] Milk Se concentration in milk consumed by lambs with elevated SGO-T in bean and Se ration was 0.194 ppm.
[3] Milk sulfur levels of ewes on the basal treatment, suckling lambs with elevated SGO-T, was 0.280 ppm.

TABLE 6

Digestion of ration Se and tocopherol and conversion to milk Se and tocopherol affected by autoclaving beans and supplemental Se

	Treatment no.			
	1	2	3	4
Apparent digestion coefficient of Se (non-lactating ewes consuming 700 g beans, 900 g hay/24 hr), %	−17.0		+6.9	
Conversion of ration and supplemental Se to milk Se, % [1]	22.5	2.06	32.0	3.75
Conversion of supplemental Se to milk Se, % [1]	—	1.56	—	1.73

[1] Conversion values calculated from milk produced during the sixth week of lactation.

way of Se excretion in ruminants is through the feces, with only a small fraction being voided in the urine.

Plant forms of Se were absorbed and incorporated into milk much more efficiently than supplemental inorganic Se (table 6). These observations are in accord with results reported by other workers (13, 14), who also noted that the major portion of plant Se is unabsorbed, appearing in the feces of sheep in an insoluble inorganic form. They concluded that reduction of dietary Se to a nonavailable form occurs in the rumen inasmuch as the predominant chemical state of Se in the plant consumed was as selenoamino acids either free or incorporated into proteins. Autoclaving aided the utilization of both bean Se and supplemental Se (table 6). There is a possibility that autoclaving beans enhances the uptake of Se before being transformed into an insoluble inorganic state.

Radioactivity of milk and tissues. The radioactivity of milk withdrawn 6 days after dosage of the ewes with ^{75}Se appears in table 7. Specific activity data revealed that as an average at equal milk Se concentrations a larger fraction of the Se was radioactive if the ewes were consuming raw beans in contrast with autoclaved beans. Radioactivity accumulated in the lamb tissues over a 6-day interval of consuming the labeled milk are recorded in table 8. The counts per minute in 15 g of lamb tissue as a fraction of the total counts per minute in the milk produced over an interval of 158 minutes are also included in table 8. The pattern of ^{75}Se distribution in lamb tissue was not dissimilar due to treatment effects, with the possible exception that more of the ^{75}Se was concentrated in the pancreas of the lambs from the raw bean ration. The pancreas has received attention as an organ sensitive to Se levels (9). Jones and Godwin (9) observed an atrophy of the acinar cells of the pancreas of mice consuming Se-deficient diets, and suggested that this atrophy may be the primary symptom of Se deficiency. Average pancreas weights in grams per kilogram of lamb were 0.81 and 0.68 for the lambs from the ewes on the autoclaved bean and raw bean treatments, respectively. Both lambs from the raw bean treatment were dystrophic but not to the degree of clinical stiffness.

It is not known whether this increased utilization of vitamin E and Se in animals fed heated beans is due to the destruction of a specific entity affecting the vitamin E and Se or due to a change in the chemical availability of the 2 nutrients.

More experimentation is required to establish preventive levels of available Se in association with given levels of vitamin E.

Lack of available Se and tocopherol may actually be a deterrent to milk production according to figures obtained in this study. However, separation of cause and effect is difficult. Does the ewe produce more milk due to the availability of these micronutrients or due to greater stimulation from lambs made healthier by their presence in the milk?

TABLE 7

^{75}Se content and specific activity of milk as affected by raw and autoclaved beans [1]

Treatment	Milk production/ 158 min	Net counts/ min/g milk	Concentration milk Se (dry matter)	Specific activity
	g	*count/min*	*ppm*	*count/min* μg Se
		Raw beans		
Ewe 1	96	227	0.048	25,021
Ewe 2	133	710	0.048	77,421
Avg	115	469	0.048	51,221
		Autoclaved beans		
Ewe 3	166	222	0.044	27,501
Ewe 4	126	133	0.048	40,318
Avg	146	178	0.046	32,910

[1] Production and radioactivity determinations 6 days after dosage with ^{75}Se.

TABLE 8

Distribution of ^{75}Se in lamb tissue and fraction in tissue as an increment of milk ^{75}Se consumed

Treatment	Blood	Liver	Muscle	Pancreas	Kidney
		count/min/15 g tissue			
		Raw beans			
Lamb 1 [1,2]	402	1451	161	1216	13,655
Lamb 2	1536	8180	482	5044	40,909
		Cooked beans			
Lamb 3	724	2430	231	1580	20,350
Lamb 4	1117	4760	288	2714	28,009
Fraction of ^{75}Se in 15 g lamb tissue as an increment of milk ^{75}Se consumed over 158 min period [1]					
		count/min $\times 10^{-3}$*/15 g tissue*			
		Raw beans			
Lamb 1 [2]	18.5	66.7	7.40	55.92	628.0
Lamb 2	16.1	85.9	5.06	52.97	429.6
Avg	17.3	76.3	6.23	54.44	528.8
		Cooked beans			
Lamb 3	19.6	65.9	6.27	42.87	552.3
Lamb 4	19.3	82.2	4.97	46.88	483.8
Avg	19.5	74.1	5.62	44.88	518.5

[1] Tissue radioactivity and fractional incorporation on basis of tissue and milk counts 6 days after dosage of ewes with ^{75}Se.
[2] Lamb numbers correspond to dam numbers in table 7.

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Skeletal Magnesium Changes in the Rat during Varying Dietary Fluoride Intake and Growth [1]

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ABSTRACT Previous work has shown that elevated intakes of fluoride by mature animals result in an augmentation of the skeletal magnesium concentration. This work describes the effects on skeletal magnesium content when 3 levels of supplemental fluoride were supplied to weanling rats over increasing time-periods. Albino rats were maintained from weanling age with fluoride intakes of either zero, 50, or 100 ppm in the drinking water for periods of 4, 8, 12, and 24 weeks. The analyses for magnesium and fluoride in the humeri indicated that the magnesium content in bone ash decreased with age whereas the total magnesium content of the bone increased. These results indicate that less magnesium was incorporated into the bone mineral resulting in an apparent dilution of magnesium as maturation of the bone occurs. The effect of fluoride at the levels used was to limit the dilution of the magnesium in bone.

Following the observation by Forbes et al. (1) that fluoride added to the rations of cows and pigs resulted in an increase in the skeletal magnesium content, several reports in the literature have shown a similar response to fluoride intake in a variety of species. Kick et al. (2) reported that growing swine given supplements of either limestone containing fluoride or of sodium fluoride had an increased fluoride and magnesium content in the skeleton and teeth as the amount of fluoride in the diet was increased. McClure and Mitchell (3) and Schulz (4) reported similar responses in albino rats. Comparing bones from rats drinking water containing 100 ppm of fluoride with those from rats drinking distilled water, McCann and Bullock (5) noted up to 10% more magnesium in bones from animals receiving the higher fluoride intake. Zipkin et al. (6) analyzed bones from humans drinking water containing from 1 to 4 ppm fluoride and demonstrated a gradual increase in the magnesium content of ribs, vertebrae and iliac crests as the fluoride intake increased.

Recently, Griffith et al. (7) studied the effects of both supplemental dietary fluoride and magnesium on the magnesium content of various tissues in growing chickens. At each of the dietary magnesium levels, fluoride supplementation resulted in an increase in the magnesium content of the bones. These authors suggested that a complex was formed between fluoride, magnesium and phosphate similar to the one proposed by Warberg and Christian (8). In reviewing the literature, it was noted that the reported studies involved relatively high levels of fluoride intake ($>$ 100 ppm) and that only the tissues of adult animals were examined for fluoride and magnesium content. It was, therefore, of interest to investigate the relationship between fluoride and magnesium in bone at different periods of animal growth and with more than one level of fluoride intake.

EXPERIMENTAL

Male albino rats [3] were started at weaning on a dietary regimen of laboratory ration [4] (0.23% Mg; 0.005 to 0.007% F) and either zero, 50 or 100 ppm F^- as sodium fluoride in the drinking water ad libitum. Twelve weanling rats were killed to provide tissues for baseline data and 10 to 12 animals on each of the 3 levels of fluoride intake were killed at intervals of 4, 8, 12 and 24 weeks. The animals were bled by heart puncture and

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[3] Holtzman Company, Madison, Wisconsin.
[4] Ralston Purina Company, St. Louis.

the humeri removed at the time they were dispatched. The bones were prepared by removing all soft tissue, reaming out the marrow cavity and fat-extracting in a Soxhlet apparatus with a 50:50 (v/v) reflux mixture of absolute ethanol and anhydrous ethyl ether for 48 hours. The fat-free bones were then dried at 100°, weighing and ashed in a muffle furnace at 550° for 8 hours. The ashed bone was weighed, powdered in an agate mortar and portions of the powdered ash were taken for fluoride and magnesium determinations. The fluoride content was determined by the microdiffusion method of Singer and Armstrong (9). Magnesium was determined with a Unicam flame spectrophotometer [5] with a modification of a method described by Alcock et al. (10). From 5 to 10 mg of bone ash were dissolved in 2 to 3 ml of dilute hydrochloric acid and the solution was evaporated to dryness. The samples were redissolved and diluted to a final volume of 25 ml with 0.1 N HCl containing 1 ml of 85% phosphoric acid/liter (14.7 mM). Standard solutions were made up to contain the same amount of phosphorus and approximately the same concentration of calcium, potassium and sodium as in the bone ash solutions. The high phosphorus concentrations in the sample solutions and standards were used to compensate for the phosphate depression effect on the excitation of the atoms.

RESULTS AND DISCUSSION

The average body weights of the animals on each of the levels of fluoride and at each time period are given in table 1. There was no significant difference in

TABLE 1

Average body weights of rats at time of killing

Weeks fed diet	F in drinking water, ppm		
	0	50	100
	g	*g*	*g*
0	34 ± 3.7 [1]	—	—
4	258 ± 20.5	236 ± 27.2	255 ± 14.3
8	384 ± 40.2	374 ± 33.3	315 ± 63.3
12	548 ± 51.1	461 ± 52.4	361 ± 25.1
24	498 ± 22.2	504 ± 21.3	372 ± 35.9

[1] SD.

[5] Model SP. 900, Unicam Instruments Ltd., Cambridge, England.

TABLE 2

Fluoride and magnesium content of the humeri of rats at various ages and fluoride intakes

Fluoride in water	Time fed diet	No. animals	Ash	Fluoride		Magnesium	
			%	*% of dry fat-free wt*	*% of ash wt*	*% of dry fat-free wt*	*% of ash wt*
ppm	*weeks*						
0	0	12	47.5 ± 1.86 [1]	0.016 ± 0.0027	0.035 ± 0.0052	0.48 ± 0.073	1.02 ± 0.116
	4	10	62.5 ± 1.05	0.037 ± 0.0051	0.055 ± 0.0080	0.58 ± 0.032	0.93 ± 0.037
	8	10	66.4 ± 0.46	0.037 ± 0.0056	0.061 ± 0.0086	0.59 ± 0.034	0.88 ± 0.032
	12	10	67.3 ± 0.56	0.046 ± 0.0102	0.068 ± 0.0152	0.56 ± 0.018	0.84 ± 0.032
	24	10	69.9 ± 0.66	0.069 ± 0.0051	0.098 ± 0.0075	0.55 ± 0.011	0.79 ± 0.016
50	4	10	62.6 ± 0.75	0.165 ± 0.0289	0.266 ± 0.0230	0.61 ± 0.023	0.98 ± 0.045
	8	10	67.7 ± 0.59	0.214 ± 0.0194	0.310 ± 0.0190	0.63 ± 0.030	0.93 ± 0.037
	12	10	67.7 ± 0.57	0.221 ± 0.0388	0.321 ± 0.0452	0.63 ± 0.025	0.91 ± 0.045
	24	10	70.2 ± 0.73	0.266 ± 0.0210	0.378 ± 0.0318	0.58 ± 0.028	0.82 ± 0.030
100	4	12	63.5 ± 1.35	0.313 ± 0.0566	0.494 ± 0.0330	0.62 ± 0.021	0.97 ± 0.032
	8	12	66.5 ± 0.98	0.389 ± 0.0432	0.584 ± 0.0670	0.62 ± 0.023	0.92 ± 0.034
	12	16	69.1 ± 1.34	0.463 ± 0.0386	0.673 ± 0.0580	0.61 ± 0.021	0.89 ± 0.029
	24	15	70.9 ± 0.66	0.581 ± 0.0410	0.820 ± 0.0630	0.59 ± 0.018	0.84 ± 0.022

[1] SD.

TABLE 3

Total magnesium in two humeri

Weeks fed diet	F in drinking water, ppm		
	0	50	100
	mg	*mg*	*mg*
0 (baseline)	0.27 ± 0.055 [1]	—	—
4	1.63 ± 0.209	1.74 ± 0.160	1.81 ± 0.232
8	2.82 ± 0.301	3.04 ± 0.203	2.91 ± 0.405
12	3.65 ± 0.547	3.66 ± 0.302	3.64 ± 0.411
24	3.75 ± 0.168	4.18 ± 0.414	3.84 ± 0.323

[1] SD.

growth of the animals receiving distilled water or water containing 50 ppm F. The growth of the animals drinking 100 ppm F was markedly depressed by the time they were killed at week 8. Random samplings of the plasma magnesium concentrations indicated little change from a value of approximately 1.0 mM as a result of the varying fluoride intake or with age of the animals. The analytical data from the humeri are summarized in table 2. The percentage ash in the humeri increased with age and neither the ingestion of 50 or 100 ppm F had an effect on the ash content of the bones. As would be expected, the fluoride content of the bones increased both with fluoride intake and with age. When expressed on a dry, fat-free basis, no significant change in the magnesium content of the bones occurred with age following a marked increase during the first 4 weeks. The magnesium content as percentage of the dry fat-free bone was higher at each of the age periods for the animals receiving either 50 or 100 ppm F than with those receiving distilled water. The magnesium content as percentage of ashed bones decreased with age regardless of the level of fluoride intake. The decrease occurring during the period from 12 to 24 weeks was highly significant ($P<0.001$) for all 3 levels of fluoride intake. Although the concentration of magnesium decreased with age, the total quantity of this element in the bone increased during the same periods (table 3). These results show that magnesium is diluted as calcification progresses and is consistent with an intracellular location of magnesium and with the amorphous calcium phosphate phase in young bone tissue. Wuthier and King [6] have also noted that the magnesium concentration in the epiphyseal cartilage of fetal calf bone decreased as mineralization occurred. The higher levels of fluoride intake limited the dilution of magnesium in calcifying bone. This effect of fluoride is indicated by the significantly higher concentration of magnesium in the bone ash of the animals receiving 50 ppm fluoride than that of the animals given distilled water at 4 weeks ($P < 0.02$), 8 ($P < 0.02$), 12 ($P < 0.02$) and 24 weeks ($P < 0.01$). Elevating the fluoride intake from 50 to 100 ppm had no further effect on the magnesium content of the bones. These results, when considered at a single period of growth, agree with the earlier reports on the effect of fluoride intake on skeletal magnesium. The increased content in bone ash due to fluoride, however, does not fully compensate for the reduction consequent on aging of the animals; for example, compare the levels at 8 weeks with those at 4 weeks. Both McCann and Bullock (5) and Griffith et al. (7) suggested that fluoride increased the affinity of bone mineral for magnesium. Our data support this concept particularly when the effect of increasing calcification of bone in causing a decrease in the incorporation of magnesium into bone is taken into account. Recent work of Pyke [7] with guinea pigs has shown that the addition of either 100 or 200 ppm fluoride to a suboptimal magnesium diet resulted in a moderation of decrease of bone magnesium. A preliminary study in our

[6] Wuthier, R. E., and P. C. King 1966 Profiles of organic and inorganic constituents of mineralizing epiphyseal cartilage and bone. International Association for Dental Research (Chicago) Abstracts, 44th General Meeting, abstr. no. 15, p. 40.

[7] Pyke, R. D., P. H. Phillips and W. G. Hoekstra 1967 Effects of fluoride on magnesium deficiency in the guinea pig. Federation Proc., 26: 415 (abstract).

laboratory indicates a similar effect of fluoride in magnesium-deficient rats.[8]

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Lethal Amounts of Casein, Casein Salts and Hydrolyzed Casein Given Orally to Albino Rats [1]

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ABSTRACT The lethal dose of casein given as an aqueous suspension intragastrically to albino rats was estimated to be well over 1000 g/kg administered over a period of 2 weeks but could not be definitely established because deaths were due in part to distilled water in the suspension. The LD_{50} of the water-soluble sodium and calcium salts of casein was estimated to be some 400–500 g/kg given over a 5-day period, the intoxication being due mainly to salt effects. The $LD_{50} \pm SE$ of pancreatin-hydrolyzed casein was found to be 26.0 ± 1.6 g/kg, death occurred at 2 to 4 hours and was due to a violent gastroenteritis, blood and tissue dehydration, widespread capillary-venous congestion, coma and respiratory failure. Survivors of the latter group recovered clinically in 2 to 3 days but some changes in organ weights were significantly abnormal at 2 weeks and even at 1 month. The results indicate it is almost impossible to administer lethal amounts of casein orally to albino rats but that toxic effects can be produced by water-soluble salts of casein and particularly by the amino acids and polypeptides of hydrolyzed casein.

During the course of studies on the toxicity of drugs given to adult albino rats fed various purified vitamin-deficient diets, evidence was obtained which suggested that toxicity may be associated with the presence of large amounts of certain foods in the diets (1). As a corollary it should be possible to demonstrate toxic and lethal doses of such foods. Certain preparations of casein were found to produce toxicity and death when given orally in sufficient amounts to albino rats. The amount required to produce death was far in excess of that likely to be fed or eaten except in the instance of hydrolyzed casein.

Hydrolyzed casein is produced in the digestion of casein with, for example, pancreatin (2). It is present in certain proprietary infant food formulas (3) and is used in the therapy of babies with special feeding problems (4) such as allergenic sensitivity to intact proteins, pancreatic deficiencies and tube feeding (it is water-soluble). The results of the present study suggest that if casein hydrolysate were fed to infants in amounts somewhat greater than the recommended 5 g/kg per day (4), it might produce toxic signs which could resemble the disease being treated. Rats may eat some 15 to 30 g/kg per day of casein (1) and these amounts of casein hydrolysate can produce lethal reactions if given to rats at one administration. The results of the present study, therefore, have important implications in the fields of animal and human nutrition.

MATERIALS AND METHODS

Techniques. Seven preparations of casein were given by intragastric cannula to overnight-starved, young, male, albino rats [2] weighing 150 to 200 g, in increasing amounts until death occurred. The animals were housed in metabolism cages, one rat per cage, and were offered laboratory ration [3] and water ad libitum. Clinical signs of toxicity were noted daily (or at shorter intervals if indicated) until the syndrome had subsided. At intervals of 24 hours measurements were made of body weight in grams, food consumption in grams per kilogram of body weight per day, water intake in milliliters per kilogram per day, colonic temperature, urinary volume in milliliters per kilogram per day, urinary

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[1] This research was supported by a grant from the Medical Research Council of Canada.
[2] The animals were a Wistar strain obtained from Canadian Breeding Laboratories of St. Constant, Quebec, Canada.
[3] Purina Laboratory Chow Checkers, Ralston Purina Company, Limited, Woodstock, Ontario, Canada.

protein and glucose output semi-quantitated in milligrams per kilogram per day, and urinary pH on a 24-hour sample. The last 3 measurements were made with the use of Ames Combistix [4] and colonic temperature was recorded by a Thermistemp Telethermometer [5] with the probe inserted into the descending colon. Other measurements were made as indicated and as noted below.

Premortem signs were recorded in detail when possible and following death the gross and microscopic appearance of the organs listed in table 1 were noted. Histopathologic studies were made of blocks of tissue fixed in Lillie's buffered formalin and sections were stained with hematoxylin-phloxine-saffron. In addition, the wet weight [6] and water content of the organs listed in table 1 were measured at death and in survivors at 2 weeks and at one month following administration of enzymatic casein hydrolysate.[7] Autopsies were performed within one hour of death to avoid the postmortem shifts in weight and water content described by Boyd and Knight (5). The contents of the lumen of the gastrointestinal organs were removed by a standardized washing and milking before weighing. The sample of muscle was the right half of the ventral abdominal wall muscle layer. The medulla oblongata was included in dissection of the brain. Water content was determined upon organs dried to constant weight at 95° in a forced-draft isotemp oven.[8] Aliquots from the dorsolumbar region of skin and from residual homogenized carcass were used for measuring water content.

Mean differences from controls were subjected to the *t* test for statistical significance (6). The $LD_{50} \pm SE$ was calculated by the linear regression method of Boyd (7).

Preparations of casein. Casein certified Fisher [9] was administered as a 30% (w/v) suspension in a 0.12% solution of ammonium hydroxide in distilled water as suggested from the data of Davies (8). Following pilot tests with lesser volumes, it was finally given warmed to body temperature in a volume of 100 ml/kg which volume appeared feasible from the report of Boyd et al. (9). This was repeated at hourly intervals to yield doses of 30, 60, 90, 120 and 150 g/kg with controls given the same volumes of 0.12% ammonium hydroxide solution, each dose of casein and of vehicle to 10 rats.

Following preliminary trials, high protein casein [10] and vitamin-free test casein [11] were administered in a dose of 50 ml/kg of a 15% (w/v) suspension at 5 successive hourly intervals each day (= 37.5 g/kg per day) for 3 days and then the dose was gradually increased to 9 administrations per day until death occurred or for 3 weeks, whichever occurred first, each preparation to 20 rats. Following similar pilot studies, sodium caseinate,[12] casein sodium and casein calcium [13] were each given as 50 ml/kg of a 15% (w/v) solution in distilled water at 10 successive intervals of 0.5 hours (= 75 g/kg per day) each day until the animals died or for one week, whichever occurred first, each preparation to 10 rats.

Enzymatic casein hydrolysate was dissolved in distilled water at body temperature and administered in a volume of 100 ml/kg in doses of 10, 20, 22.5, 24, 25, 26,

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[4] Manufactured by the Ames Company of Canada Limited, Rexdale, Ontario, Canada.

[5] Purchased from the Fisher Scientific Company Limited, Don Mills, Ontario, Canada.

[6] Organs were weighed to 0.1 mg on a 1-911X3 Mettler semi-micro gramatic balance, except skin and residual carcass which were weighed on a Mettler K-5T precision balance, both balances purchased from Fisher Scientific Company Limited, Don Mills, Ontario, Canada.

[7] Enzymatic casein hydrolysate is produced by treating casein with pancreatin which converts casein into its amino acids and peptides. General Biochemicals, Chagrin Falls, Ohio, from whom it was purchased, state that it is edible and that the degree of hydrolysis is "acceptable to the American Medical Association." The preparation was found to be soluble to 60% (w/v) in distilled water at body temperature.

[8] See footnote 5.

[9] See footnote 5.

[10] High protein casein was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it was produced by the controlled lactic acid fermentation of pure skim milk and contained 85% protein, 11% water, 1.9% ash, 1.5% milk fat and small amounts of thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid, cyanocobalamin, copper, iron, zinc and iodine.

[11] Vitamin-Free Test Casein was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it is prepared by controlled multiple extractions with hot alcohol and vacuum drying and contains 89.0% protein, 8.0% water, 2.0% ash and 0.5% fat. Alcohol extraction removes some 75% (35 to 100) of the vitamins contained in high protein casein so that it is not "vitamin-free" but may be considered such for most biological purposes.

[12] Sodium caseinate was obtained from General Biochemicals, Chagrin Falls, Ohio, who reported that it contained 92.5–94.5% protein, 4.0% ash (1.3% sodium) and 1.5% fat. It was found soluble to 20% in water at body temperature.

[13] Casein sodium and casein calcium were obtained from Nutritional Biochemicals Corporation, Cleveland. Casein sodium was found soluble in water to 25% and casein calcium to 15% at body temperature.

27.5, 29, 30, 40, and 50 g/kg each dose to 16 to 20 rats with 31 controls given 100 ml/kg of distilled water only.

RESULTS

Casein. Suspensions of casein Fisher gradually coagulated into particles of various sizes on standing and also in the rat's stomach. A dose of 30 g/kg killed 30% of the rats by regurgitation and aspiration into the lungs where it produced death from aspiration asphyxia, usually within 30 minutes, or from gastric rupture with death at 6 to 14 hours later. The LD_{50} of ammonium hydroxide, given in solution to the controls, was found to be 0.45 g/kg; death followed convulsoins at 0.5 to 4 hours and was accompanied by a violent gastritis. The LD_{50} of ammonium hydroxide is similar to that reported previously in cats by Spector (10).

High protein and vitamin-free test casein suspensions produced 40% deaths from stomach rupture after administration of 37.5 g/kg (day 1). There were no further deaths until the eighth day when daily administrations had increased to seven, or 52.5 g/kg per day, and a total of 345 g/kg of casein had been given — at which point 50% of rats had died of gastric rupture. Twenty percent of the rats survived 9 administrations or 67.5 g/kg per day for one week following the fourteenth day when this daily dose was reached. The clinical signs in these 8 rats were inhibition of growth, anorexia, a marked diuresis due largely to daily administration of large volumes of water, proteinuria, aciduria, and listlessness. These signs were accentuated premortally in 3 rats that died apparently of protein intoxication and at least not from gastric rupture or from regurgitation asphyxia.

Ability to survive large amounts of "natural" casein (high protein casein, vitamin-free test casein, and casein Fisher) appeared to be related mainly to ability of the stomach to accommodate the necessary large bulk. In survivors there was some capillary-venous congestion of the lamina propria and submucosa of the stomach, small bowel, cecum and colon. There was some evidence of systemic damage. The liver was congested and occasionally there were scattered areas of necrosis in the lobules. There was mild-to-moderate capillary-venous congestion of the brain, lungs, heart and kidneys and atrophic changes in the myofibrils of skeletal muscle and in the thymus gland. These changes were accentuated in 3 rats that died apparently of casein poisoning. The total dose of casein given to the latter 3 rats was 140, 340 and 830 g/kg given over 4, 8 and 15 days, respectively. If calculation of the LD_{50} can be applied under these circumstances, casein would have an LD_{50} well over 1000 g/kg.

Casein salts. Of the 10 rats given sodium caseinate, 2 died of gastric rupture during the first 24 hours and 3 died on the fourth and fifth days with no gastric rupture and no evidence of regurgitation asphyxia. The latter 3 animals had received 75 g/kg per day of sodium caseinate (containing approximately 1 g/kg per day of sodium) or a total of 300 to 375 g/kg over the interval to death. The indicated "LD_{50}" would be of the order of 400 to 500 g/kg. Death was preceded by marked loss of body weight, marked anorexia, marked diuresis, marked proteinuria, alkalinuria, diarrhea, listlessness and a premortal hypothermia. The local inflammatory reaction in the gut was confined mostly to the cecum. There occurred hepatic, renal, cerebral, cardiac, pulmonary, testicular, adrenal, thymic and splenic capillary-venous congestion and fatty degeneration of the renal tubules. Animals that survived had very marked diuresis and an augmented water intake.

Results in animals given casein sodium were similar to those obtained from sodium caseinate. Of the 10 rats given casein calcium, 8 died of stomach rupture during the first and second days and the reaction of the 2 survivors was similar to that seen in rats which survived administration of sodium caseinate.

Casein hydrolysate. The $LD_{50} \pm SE$ of enzymatic casein hydrolysate was found to be 26.0 ± 1.6 g/kg, the maximal LD_0 was 23.5 and the minimal LD_{100} was 28.6 g/kg. The mean $\pm$ SD interval to death was 3.6 ± 1.4 hours excluding one delayed death at 24 hours and a second at 27 days. The interval to death was shorter the higher the dose of the casein hydrolysate.

Clinical signs of toxicity during the first hour were listlessness, cyanosis, and diarrheic bowel movements which appeared

to consist mostly of casein hydrolysate. Hemoconcentration developed rapidly as shown in figure 1 and was greater the higher the dose of the casein hydrolysate as shown in figure 2. Death was due to respiratory failure in a cyanotic coma. At autopsy, most organs were found to have lost weight, as shown in table 1, due to loss of water, as shown in table 2. On gross observation, an intense congestion of the brain, hemorrhagic inflammation of the gut and a dark-colored liver were observed. Microscopic examination confirmed the gross pathology and disclosed vascular congestion in many other organs as indicated in table 3 and, in the delayed death, degenerative changes in the kidneys and lungs. Blood clots, noted in table 3, were due either to antemortem clotting or to accelerated postmortem clotting or to both, since they were not seen in controls given no casein enzymatic hydrolysate.

Survivors at 24 hours had lost body weight and exhibited anorexia, hyperdipsia, mild fever, diuresis, aciduria and proteinuria but appeared grossly normal, as indicated by results summarized in table 4. The data in table 4 are expressed as percentage of change from controls given 100 ml/kg of distilled water which in itself produced a moderate diuresis, alkalinuria, glycosuria and proteinuria. By the third day, clinical parameters were returning toward normal but not all organ weights and water

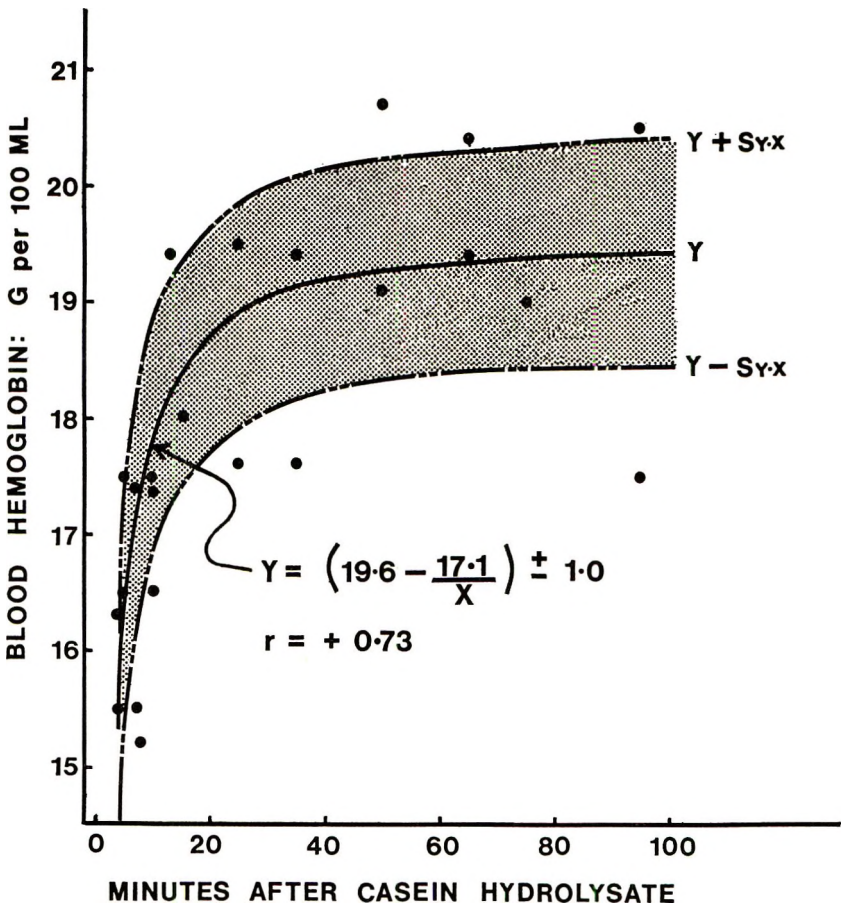

Fig. 1 The regression, on time in minutes, of values for blood hemoglobin following oral administration of casein enzymatic hydrolysate in a dose of 26 g/kg body weight.

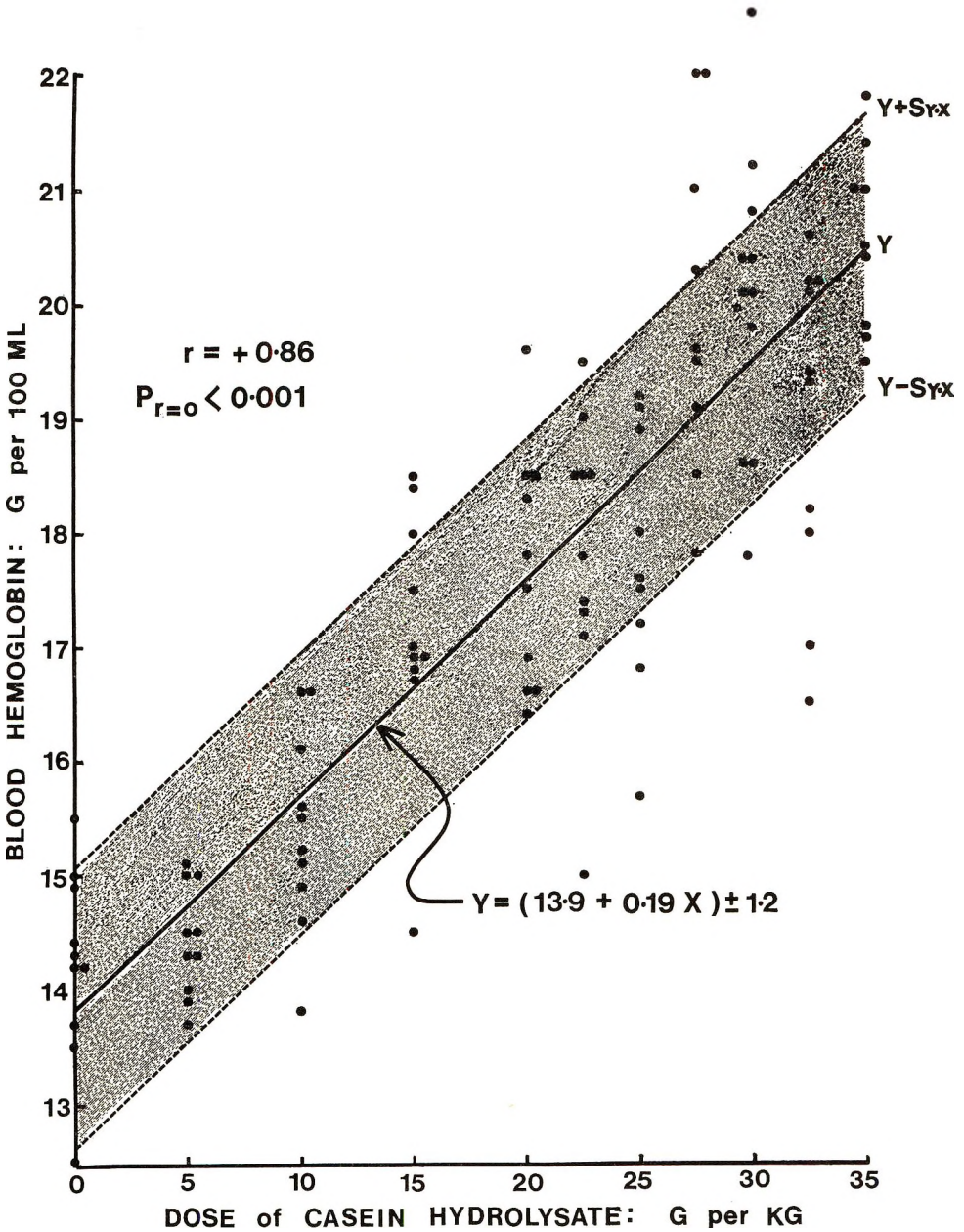

Fig. 2 The regression, on dose of casein enzymatic hydrolysate of values for blood hemoglobin measured at 30 minutes later.

content had reached normal values at 2 weeks and even at one month as indicated by data summarized in tables 1 and 2. All clinical parameters were normal at 2 weeks and one month.

DISCUSSION

The results from casein administration confirm conclusions reached by Bischoff (11) in his review of 1932 and by Hegsted (12) in 1964 that man and animals can

TABLE 1

Changes in the fresh weight of body organs at autopsy in albino rats given doses of casein enzymatic hydrolysate in the range of the oral LD_{50}[1]

Organ	At death (N = 17 + 19 controls)	2-week survivors (N = 15 + 14 controls)	1-month survivors (N = 18 + 16 controls)
Adrenal glands	− 4.3	− 3.8	−12.5 **
Brain	− 3.2 *	− 0.3	+ 0.1
Gastrointestinal tract:			
Cardiac stomach	−24.1 **	−17.7 *	+ 2.0
Pyloric stomach	−21.2 **	− 8.8	− 0.7
Small bowel	− 5.2	− 9.2	+ 9.5 **
Cecum	−19.7 **	+ 4.9	− 0.1
Colon	−18.9 **	−10.8 *	+ 3.6
Heart	− 2.4	+ 2.9	− 3.4
Kidneys	− 5.2 *	− 5.9 *	+ 4.0
Liver	− 6.6	− 5.0	+ 8.4 *
Lungs	+13.6	− 7.6 *	+ 1.5
Muscle (ventral abd. wall)	−30.8 **	− 3.6	−17.5 **
Salivary glands (submax.)	− 2.3	+ 1.5	− 0.6
Skin	−11.2 **	− 0.5	− 2.9
Spleen	−28.4 **	− 8.5	− 7.8
Testes	−17.1 **	− 2.3	− 3.0
Thymus gland	−17.4 *	−11.4 *	− 7.9
Residual carcass	−13.7 **	− 3.7 *	− 0.2
Total body wt	− 2.4	− 3.2 *	− 0.7

[1] The organs were weighed in grams and the results are expressed as mean percent change from controls, specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated rats and $\bar{X}_c$ in the respective controls.

* A mean difference significantly different from zero at $P = 0.05$ to 0.02.

** A mean difference significantly different from zero at $P = 0.01$ or less.

TABLE 2

Changes in the water content of body organs at autopsy on albino rats given doses of casein enzymatic hydrolysate in the range of the oral LD_{50}[1]

Organ	At death (N = 17 + 19 controls)	2-week survivors (N = 15 + 14 controls)	1-month survivors (N = 18 + 16 controls)
Adrenal glands	−24.6 **	+8.3 *	+7.6
Brain	−15.2 **	−0.3	+0.2
Gastrointestinal tract:			
Cardiac stomach	−41.0 **	−5.3	−0.2
Pyloric stomach	−41.9 **	−2.6	+3.2
Small bowel	−30.3 **	−0.6	+0.8
Cecum	−24.6 **	+0.9	+2.5
Colon	−33.2 **	+1.0	+3.9 *
Heart	−17.3 **	−0.6	−0.2
Kidneys	−17.8 **	−0.7	−0.4
Liver	−17.0 **	+0.5	−6.0 *
Lungs	−16.0 **	−2.2	+1.2
Muscle (ventral abd. wall)	−24.0 **	+0.9	−1.0
Salivary glands (submax.)	−16.2 **	+0.6	+2.5
Skin	−23.6 **	−2.2	−2.7
Spleen	−11.3 **	−0.7	+0.7
Testes	−17.6 **	+1.6 *	+0.6
Thymus gland	−19.5 **	−2.2	+2.3
Residual carcass	−21.3 **	−4.8 *	+3.0

[1] Water content was measured as grams water/100 g dry weight of tissue and the results are expressed as mean percent change from controls, specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated rats and $\bar{X}_c$ in the respective controls.

* A mean difference significantly different from zero at $P = 0.05$ to 0.02.

** A mean difference significantly different from zero at $P = 0.01$.

TABLE 3

Histopathologic observations in albino rats at death due to oral administration of a lethal dose of casein enzymatic hydrolysate

Organ	Histopathology
Adrenal glands	Sinusoidal erythrocytes packed and distorted; clotting; minute areas of early necrosis
Brain	Marked capillary-venous congestion and hemorrhages in the meninges and brain
Gastrointestinal tract:	
Cardiac stomach	Capillary-venous congestion of the submucosa with areas of lysis of the stratified squamous epithelium
Pyloric stomach	Capillary-venous congestion of the lamina propria and submucosa
Small bowel	Capillary-venous congestion of the lamina propria and submucosa and shrunken villi
Cecum	Capillary-venous congestion and hemorrhage of the lamina propria and submucosa and lysis of glands
Colon	Capillary-venous congestion of the lamina propria and submucosa
Heart	Coronary capillaries and veins congested and occasionally blood clots present
Kidneys	Vascular congestion especially in the loop region and tubular fatty degeneration in late deaths
Liver	Sinusoids packed with distorted erythrocytes and areas of venous clotting
Lungs	Venous clots in early deaths and areas of edema and hemorrhage in late deaths
Muscle	Fibers shrunken but otherwise normal in appearance
Salivary glands (submax.)	Normal appearance
Skin	Ischemic
Spleen	Red pulp shrunken, packed erythrocytes, venous clots
Testes	Tubules shrunken, extravascular clots, some tubular lysis
Thymus gland	Venous clots and some loss of thymocytes

tolerate large amounts of casein in their diets. Hegsted (12) notes that a high protein diet increases the need for water. When young rats in this laboratory (13) were fed a diet of 80% casein for 14 days, there occurred a marked diuresis, some generalized body organ dehydration and an increase in the weight of kidneys and liver but the appearance and growth of the animals were normal.

In the present study, an attempt was made to find a lethal dose of casein given by intragastric cannula to albino rats. Aqueous suspensions coagulated unless they were relatively dilute. The dilute suspensions coagulated and remained in the stomach and repeated administration produced gastric rupture or regurgitation asphyxia in 62 out of 70 animals. Of the 8 survivors, three died apparently from casein intoxication and calculations suggested that the "LD_{50}" of casein would be greater than 1000 g/kg administered over 15 days. Administration of such amounts required the simultaneous administration of distilled water in amounts up to 450 ml/kg per day. The separate administration of this daily volume of water produced clinical signs of toxicity similar to those seen in rats given casein except that there was no aciduria nor any deaths.[14] The widespread vascular congestion of body organs seen in rats dying apparently of casein intoxication was not seen in rats which died

[14] These results were obtained by S. J. Liu of this laboratory in current studies on the effects of daily administration of large volumes of distilled water to albino rats.

TABLE 4

Clinical measurements on survivors of death due to oral administration of casein enzymatic hydrolysate [1]

Measurement	Days after casein		
	1	2	3
	% change		
Body wt, g	− 3.3 ** DD	− 4.1 ** DD	− 1.8 *
Food intake, g/kg/24 hr	− 44.5 **	− 4.9	+ 0.2
Water intake, ml/kg/24 hr	+ 80.8 ** DD	+ 21.2 **	+ 3.6 *
Colonic temperature, °C	+ 0.7 ** DD	+ 0.4	+ 0.1
Urinary volume, ml/kg/24 hr	+ 82.3 ** DD	+ 35.8	+16.8
Urinary pH, 24-hr sample	− 4.4 **	− 0.2	−10.1
Urinary glucose output, mg/kg/24 hr	−100.0 **	+109.0	+36.1
Urinary protein output, mg/kg/24 hr	+494.0 ** DD [2]	− 6.0	−53.2 *
Listlessness, cyanosis, diarrhea, clinical units	0.0	0.0	0.0

[1] The results are expressed as mean percentage change from controls specifically as $((\bar{X}_d - \bar{X}_c) \div \bar{X}_c) \times 100$ where $\bar{X}_d$ is the mean in the drug (casein) treated survivor and X_c in the controls. Dose-dependence of the mean percentage change is indicatd by "DD."

[2] Urine during the first 24 hours was contaminated with diarrheal casein hydrolysate which may have contributed to the markedly increased output of urinary protein.

* $\bar{X}_d - \bar{X}_c$ significantly different from zero at $P = 0.05$ to 0.02.

** $\bar{X}_d - \bar{X}_c$ significantly different from zero at $P = 0.01$ or less.

of acute water intoxication (14). Death in rats given casein was due to a combination of the toxic effects of water and casein and the LD_{50} of casein is undoubtedly much higher than 1000 g/kg administered over 2 weeks.

The LD_{50} of the soluble sodium and calcium salts of casein was estimated to be some 400 to 500 g/kg given orally to rats over 5 days. The signs of intoxication included some which have been reported in water (14) and in sodium chloride (15) intoxications, mainly in the latter. If large amounts of this preparation were fed in substitution for casein it could produce toxic effects due only in part (if at all) to its casein component.

When casein is hydrolyzed into its component amino acids and polypeptides, it becomes water-soluble and osmotically active. When doses of the order of 25 g/kg were given orally to albino rats in concentrated aqueous solution, they produced a violent gastroenteritis, a withdrawal of water from tissues and blood, widespread capillary-venous congestion, coma and death within a few hours. The syndrome is similar to that found in acute poisoning from sucrose (16) and from concentrated solutions of raw egg white (9). It is obvious that when this preparation is substituted for casein, it should be given in amounts well below the toxic level.

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Dietary Production of Congenital Copper Deficiency in Swine [1]

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ABSTRACT A method for the production of copper deficiency in the adult sow and newborn pig is described. Attempts to produce neonatal deficiency in the pig by the depletion of normal maternal copper reserves through repeated breeding with a copper-deficient ration were unrewarding. It was necessary to raise gilts from birth to maturity in a copper-depleted state before copper deficiency was observed in their offspring. Brain and liver copper levels as low as 2.3 ± 1.5 and 2.4 ± 0.9 μg/g dry weight, respectively, and serum copper levels of 9 ± 3 μg/100 ml were achieved in the newborn deficient animals. These animals were asymptomatic and lacked gross lesions but manifested a slight reduction in tensile strength of the aorta and a rapid development of anemia in early postnatal life.

Naturally occurring copper deficiency associated with lesions in the nervous or cardiovascular systems has been reported in lambs and kids with swayback (1, 2), in cattle with falling disease (3), in pigs with paresis (4–6) and in the red deer with ataxia (7). The effects of deficiency are manifested in utero in the lamb and goat, while in cattle, the pig and red deer they first appear in maturing or mature animals. In addition, extensive and detailed studies have been made of experimentally induced copper deficiency in several species. From these experiments there have resulted descriptions of anemia in rats (8) and swine (8–11), skeletal changes in dogs (12) and swine (13) and cardiovascular lesions in swine (14), chicks (15–17) and rabbits (18). All of these studies were made on animals rendered deficient after birth; relatively few studies have been made of the production of copper deficiency in the fetus. Copper deficiency has been produced in the newborn rat and has resulted in decreased viability, anemia, edema, hemorrhages and abdominal hernias (19). Attempts to produce swayback experimentally have been indirect and have met with equivocal results (20, 21). Recent studies with guinea pigs have indicated that congenital copper deficiency may be associated with alterations in the nervous system.[2,3] Our studies of the effect of copper deficiency on the nervous system of the lamb (1, 22–24) and on the cardiovascular system of swine (14) have led us to a closer examination of the role of this metal in the fetus. The present study records our observations on the dietary production of maternal and fetal copper deficiency in swine.

MATERIALS AND METHODS

The animals selected for this study were a breed of miniature pig (25). Sows were of the Pitman-Moore strain [4] and the boars were of the Hanford strain.[5]

The first experiment was designed to induce copper deficiency in the adult sow by a combination of low copper diet and drain on copper stores from repeated gestations. Two sows were started with the experimental diet in the last half of pregnancy. Alterations were made in the diet at various periods until an acceptable, low copper-containing mixture was achieved, which would sustain pregnancy. These al-

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[2] Tsai, M. D., G. J. Everson and R. Shrader 1964 Copper deficiency in the guinea pig. Federation Proc., 23: 133 (abstract).

[3] Everson, G. J., and Tong-In Wang 1967 Copper deficiency in the guinea pig and related brain abnormalities. Federation Proc., 26: 633 (abstract).

[4] Vita-Vet Labs, Marion, Indiana.

[5] We thank Dr. L. Bustad and Dr. H. Ragan of Richland, Washington, for these animals.

TABLE 1

Manipulation of daily diet and duration of feeding during sequential pregnancies of sows (exp. 1)

	Days fed diet [1]						
	58	12	44	58	108	39	53
Milk undiluted, ml [2]	1620	2430	1620	1600	1600	1600	1600
Total solids, g	421	632	421	416	416	416	416
Glucose monohydrate, g [3]	—	—	—	200	200	200	200
Sucrose, g	300	125	150	—	—	—	—
Sulphide water, ml [4]	ad lib.	ad lib.	ad lib.	ad lib.	ad lib.	1600	2400
Cellulose, g [5]	400	600	400	—	—	—	—
Nonnutritive fiber, g [6]	—	—	—	250	200	100	100
Vitamin supplement, g [7]	18.6	7.8	9.3	2	2	1	1
NaCl, g	6	10	6	6	6	2	4
Calcium glycerophosphate, g [8]	—	30	—	20	—	—	—
Magnesium sulphate, g [9]	2	8	2	2	3	—	—
Iron supplement, ml [10]	1	1	1	1	1	5	5
Mineral supplement, ml [11]	4	4	4	4	4	5	5
Copper supplement, ml [12,13]	10	10	10	10	10	5	5

[1] Time starts in middle of first pregnancy.
[2] Carnation Evaporated Milk, Carnation Company, Los Angeles; 100 ml contained: (in grams) protein, 7.4; fat, 8.4; carbohydrates, 10.5; minerals, 1.6; and moisture, 78.1.
[3] Cerelose, Corn Products Company, New York.
[4] Ten milliliters of a 0.36% solution of sodium sulphide in 39 liters of tap water; allowed to stand for 24 hours.
[5] Alphacel, Nutritional Biochemicals Corporation, Cleveland.
[6] General Biochemicals Inc., Chagrin Falls, Ohio.
[7] Vitamin diet fortified, vitamin D omitted; 1 kg supplied (in grams): vitamin A conc, 4.5; α-tocopherol, 5; ascorbic acid, 45; inositol, 5; choline chloride, 75; menadione, 2.25; *p*-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamine·HCl, 1; Ca pantothenate, 3; (in mg) biotin, 20; folic acid, 90; and vitamin B_{12}, 1.35; obtained from Nutritional Biochemicals Corporation.
[8] Nutritional Biochemicals Corporation.
[9] Mallinckrodt Chemical Works, St. Louis.
[10] Carbonyl iron powder, Antara Products, General Aniline and Film Corporation, New York; 72 mg/ml, 72 g powder added slowly to 330 ml reagent grade concentrated HCl. Deionized water added to dissolve the salt and make volume up to 1 liter.
[11] Reagent grade salts in deionized distilled water: (in grams/liter) manganese chloride, 1.8; aluminum sulfate, 0.6; sodium fluoride, 3.1; zinc sulfate, 1.8; cobalt nitrate, 1.8; and nickel acetate, 2.8.
[12] Two grams of copper sulphate/liter of deionized distilled water.
[13] Control sow only.

terations are summarized in table 1. The animals were housed in specially constructed stainless steel pens with slotted oak floors coated with an epoxy resin. Stainless steel equipment was used to prepare and feed the diet. The sows were rebred after each gestation. Two litters were obtained from the control sow and 3 litters from the experimental sow on this regimen. The newborn animals were bled for serum copper determinations and then slaughtered for liver and brain copper analyses.

The second experiment was designed to assure a copper-poor state in the sow by rearing the animals from birth with a copper-deficient diet and maintaining them with the diet through each gestation. The animals were weaned to a standard milk diet at 4 days of age and placed in individual pens constructed of galvanized iron fitted with stainless steel feed trays. The diet consisted of equal parts of canned evaporated milk and sulphide water fed at the rate of 230 ml/kg per pig a day supplemented from day 7 with iron and minerals, prepared as in table 1, in the amount of 0.5 ml and 0.2 ml/kg per pig a day, respectively, to a maximum of 5 ml/day. Control animals received the copper supplement at a rate of 0.5 mg/kg per pig a day to a maximum of 2.5 mg. Two control sows, two deficient sows and a control sow transferred to a deficient diet at 30 days of age formed the experimental group. The animals were weighed weekly, at which time blood samples were taken for serum copper analyses and determination of the volume of packed red cells (VPRC). The deficient animals received a 2.5 mg copper supplement when the VPRC approached 30% or the serum copper level was below 40 μg/100 ml. After each animal reached 10 kg in weight, additions to the basic milk-sulphide water mixture fed daily were in increments of 120 ml/kg per pig. The

TABLE 2

Daily diet for sows (exp. 2)

	6 months to 1 year of age	Last 6 weeks of first gestation	Maintenance diet [1]
Milk undiluted, ml [2]	2400	2400	1600
(Milk, total solids, g)	(624)	(624)	(416)
Sulphide water, ml [2]	2400	3000	1600
Glucose monohydrate, g [2]	400	450	400
Nonnutritive fiber, g [2]	50	50	25
NaCl, g	2	2	3
Vitamin supplement, g [2]	2	2	4
Iron supplement, ml [2]	5	5	5
Mineral supplement, ml [2]	5	5	5
Copper supplement, ml [2,3]	5	5	5

[1] Restricted diet supplies 1 mg copper/day; supplemented diet supplies 3.5 mg copper/day.
[2] Prepared or as described in table 1.
[3] Control sows only.

TABLE 3

Copper content of blood and tissues of newborn pigs from successive litters of sows fed the control or deficient diet (exp. 1)

	Litter no.	Serum copper	Brain copper	Liver copper
		μg/100 ml	*μg/g dry wt*	*μg/g dry wt*
Control	1	164 [1] ± 13 [2] (7) [3]	18 ± 0 (2)	146 ± 14 (2)
	2	55 ± 15 (6)	14.8 ± 1.3 (4)	160 ± 39 (4)
Deficient	1	132 [1] ± 7 (5)	19 ± 1 (2)	196 ± 19 (2)
	2	83 ± 2 (5)	17 ± 0 (2)	168 ± 22 (2)
	3	40 ± 8 (9)	17.2 ± 2.1 (7)	100 ± 21 (7)

[1] Whole blood copper.
[2] Mean deviation (arithmetic average of all the differences between the observations and their mean).
[3] Numbers in parentheses indicate number of animals examined.

diet was judged adequate by a steady weight gain and any leveling off of weight was balanced by a dietary increase. Vitamin supplements, nonnutritive fiber, sodium chloride and glucose monohydrate [6] were added from 4 months of age in successive months until the diet in table 2 was achieved at 6 months of age. When the animals were near maturity they were transferred to the larger, specially constructed pens described in the first experiment. By this method the animals were raised to sexual maturity in a copper-depleted state and subsequently bred.

Two litters were obtained from each of the copper-deficient animals and one litter was obtained from each of the control sows and the control sow transferred to a deficient diet at 30 days of age. Newborn animals were bled for serum copper and VPRC determinations and then killed for liver and brain copper analyses. Three piglets from the second litter of a copper-deficient sow were weaned onto the standard milk-sulphide water diet at 4 days of age and killed at intervals up to 25 days of age for tissue copper determinations. Complete autopsies were performed. Segments of descending thoracic aorta and loops of skin from the hind limb just proximal to the foot were obtained for tensile strength measurements (26–28). The samples for copper analyses were examined with a Perkin-Elmer model 303 atomic absorption spectrometer.[7]

RESULTS

In the first experiment, both the control and the experimental animals were maintained in good condition. The newborn young were generally active and, except for an occasional runt, were of normal weight. There was a reduction in the liver copper content and serum copper of the third litter of the depleted sow but apart from this, the brain, serum and liver copper values were unchanged (table 3).

[6] Cerelose, Corn Products Company, New York.
[7] Unpublished data, N. Weissman and B. J. Lythgoe.

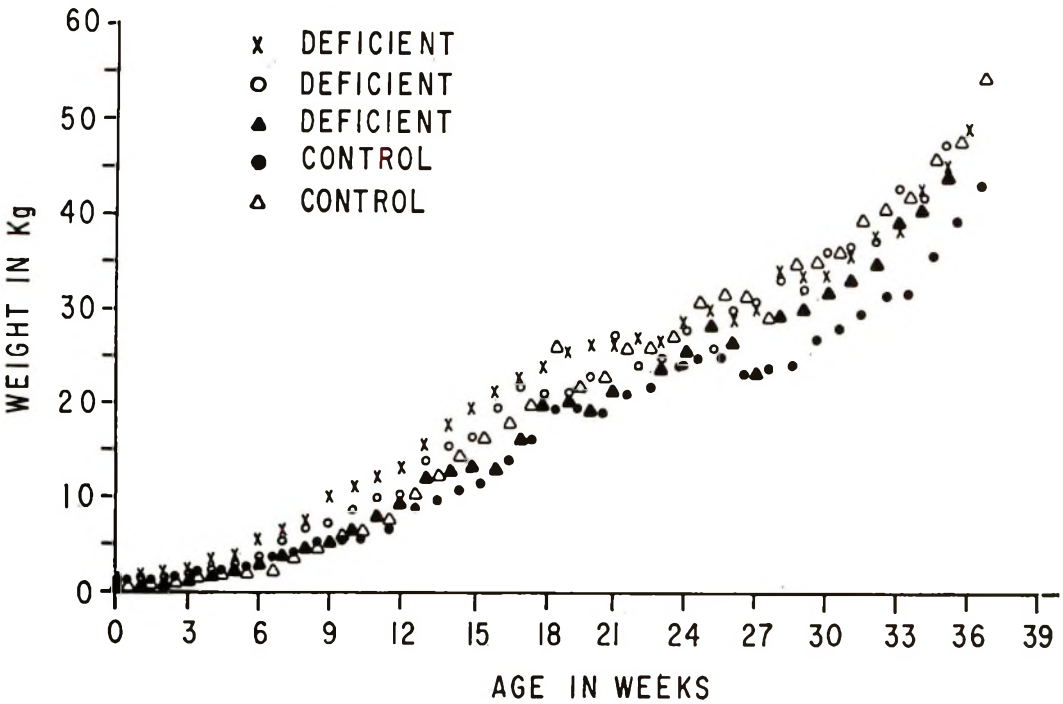

Fig. 1 Growth of animals in experiment 2. The animal indicated by X was a control transferred to a deficient diet at 30 days of age.

In the second experiment, the gilts of both the copper-supplemented and copper-restricted groups showed similar weight gains during the first 36 weeks of life (fig. 1). The details of the serum copper determinations of each of the animals from this same period are shown in figure 2. The decline in serum copper in the two deficient animals began at 4 weeks of age and reached the extremely low values of 6 and 9 μg/100 ml by 8 weeks of age. The copper-supplemented animal that was reversed to a deficient diet at one month of age reached similar low serum copper levels at almost the same time. Eight supplements of copper were administered to the animals fed the deficient diet from the ninth to thirty-fourth week of age. The animals have been followed for an additional year with the diet and no further copper supplementation has been required to maintain a serum copper level in the range of 10 to 30 μg/100 ml in the deficient animals and 20 to 50 μg/100 ml in the control sow transferred to the deficient diet at 30 days of age. The VPRC has been between 35 and 45 in all the animals.

Each gilt farrowed its first litter between 12 and 13 months of age. The newborn animals were of normal weight, generally active, and did not appear abnormal. The copper content of the brain, liver and serum of the piglets derived from the copper-restricted sows was significantly reduced compared with that of the offspring of the supplemented animals (table 4) and all the newborn of experiment 1.

Newborn animals maintained with a deficient diet for up to 25 days developed severe anemia associated with severe copper deficiency (table 5). No consistent alteration was noted in the tensile strength of the aorta and skin although the lowest values were found in the deficient animals (table 6).

DISCUSSION

Milk or milk products are the base in many of the diets used in the production of experimental copper deficiency (8–11,

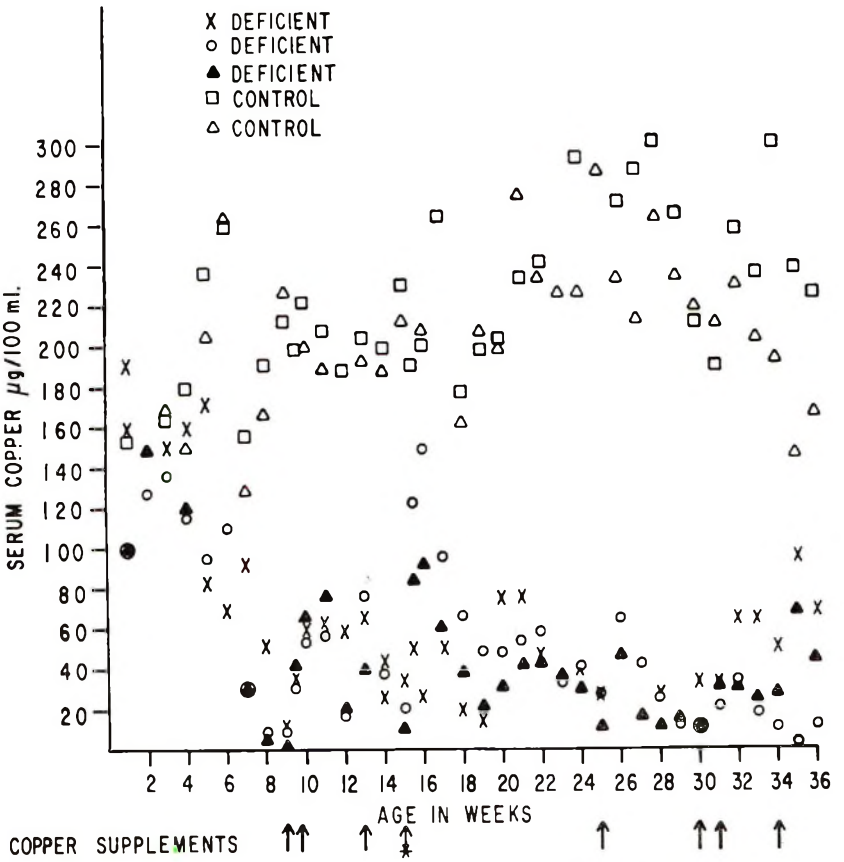

Fig. 2 Development and maintenance of a copper-deficient state in growing animals (exp. 2). The copper supplements were 2.5 mg except for one supplement of 7.5 mg. The animal indicated by X was a control transferred to a deficient diet at 30 days of age.

TABLE 4

Copper content of blood and tissues of newborn pigs from successive litters of sows fed the control or deficient diet from 4 days of age (exp. 2)

	Litter no.	Serum copper	Brain copper	Liver copper
		µg/100 ml	*µg/g dry wt*	*µg/g dry wt*
Control	1	43.3±5[1] (6)[2]	15.3 ±1 (4)	117 ±18 (4)
Deficient A[3]	1	24.5±8.5 (4)	5.4 ±1.2(5)	4.4± 1.3(5)
Deficient B	1	—	7.5 ±0.4(5)	4.8± 0.6(5)
	2	11.3±2.4 (5)	7.5 ±0.6(6)	5.9± 1.8(5)
Deficient C	1	19 ±5.7 (6)	4.0 ±0.8(6)	3.3± 1.3(6)
	2	9 ±3 (2)	2.35±1.5(2)	2.4± 0.9(2)

[1] Mean deviation (arithmetic average of all the differences between observations and their mean).
[2] Numbers in parentheses indicate number of animals examined.
[3] Control sow transferred to a deficient diet at 30 days of age.

13–19). Although these diets are low in copper they are effective mainly in young animals when the demands of growth for copper together with the low dietary supply combine to deplete the available tissue reserves. This is well-demonstrated by our experiments in which significant neonatal copper deficiency was produced only in the litters of sows reared from birth with a copper-deficient diet. Despite the stress of 3 gestations it was not possible to deplete the copper stores of a commercially bred

TABLE 5

Terminal copper content and volume of packed red cells (VRPC) in piglets maintained beyond birth

Age at killing	VPRC	Serum copper	Brain copper	Liver copper
days [1]	%	*μg/100 ml*	*μg/g dry wt*	*μg/g dry wt*
14	20	3.2	2.8	2.9
19	10	19.4	2.9	1.75
25	10	19.8	1.8	1.4

[1] One animal at each age.

TABLE 6

Tensile strength of aorta and skin of newborn piglets

Group	No. of animals	Aorta	Skin
		kg/cm²	*kg/cm²*
Control	4	22.5 ± 7.6	50.2 ± 14.0
Deficient A1	7	13.9 ± 6.1	49.4 ± 12.0
B1	6	18.0 ± 3.9	35.9 ± 5.4
C1	6	12.2 ± 6.2	48.2 ± 14.8

adult sow fed the milk diet. In our second experiment the deprivation of the dietary copper from birth in the breeding stock has been shown to be effective in producing congenital copper deficiency. Such congenital deficiency has been asymptomatic and without gross cardiovascular or central nervous system lesions though deficient pigs reared for a short time developed severe anemia. This suggests that the fetal copper status was sufficient to sustain development but not the rapid growth of early extra-uterine life.

The anemia ascribed to copper deficiency is generally associated with a severe depletion of copper reserves and serum copper levels near 20 μg/100 ml (10, 11). The sows raised from birth in a copper-deprived state and their offspring were near this level. However, anemia was not observed in our animals. That such a condition was imminent is indicated by the rapid development of progressive anemia in the newborn animals maintained beyond birth.

Studies on the mechanical properties of the aorta of copper-deficient and control swine before and after elastin isolation and digestion have demonstrated a reduction of the tensile strength of the aorta from deficient animals (26–28). This abnormality has been attributed to altered elastin. In our experiments the values for the tensile strength of the aorta in the newborn deficient animals were generally lower than those in the controls. Although these data suggested the presence of altered elastin the values obtained were not low enough to be statistically significant. It is possible that the degree of copper deficiency was insufficient to interfere with elastogenesis although similar serum copper levels are usually associated with severe cardiovascular lesions in older swine (14). The tensile strength of the skin of most of the copper-deficient animals was within the normal range although in one group of deficient animals the tensile strength was low. The significance of this last observation will have to await further investigation. In previous studies no differences have been noted in the tensile strength of skin from older copper-deficient and control animals (28).

In both experiments all females had equal breeding opportunities but the copper-deprived stock in each experiment produced more litters than the control stock over the same period. In such small groups this difference may be coincidental, but considered in the light of the deleterious reproductive effects of copper deficiency in the rat (29) this result was unexpected.

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Degradation and Excretion of Riboflavin in the Rat [1,2]

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ABSTRACT Carbon-14-labeled riboflavin was injected intraperitoneally into young adult rats to study the degradation and excretion of the vitamin. The distribution of radioactivity 24 hours after administration was found to be high in the liver and kidney but very low in the blood. Almost all the radioactivity administered was recovered from urine, feces, and carcass at the end of the experiment. The radioactivity of these samples was due mainly to intact riboflavin, and except for lumichrome and lumiflavin no other degradative product of riboflavin was found. The ribitol side chain on position 9 of riboflavin was not degraded in the tissues. However, there was low production of lumichrome, lumiflavin, and CO_2 from riboflavin by the intestinal microorganisms. The turnover rate of this vitamin in the body was affected by the level of its intake, a half-life of 16 days being observed for rats under normal physiological conditions. The maintenance requirement of riboflavin for the rat was shown to be dependent mainly upon excretion rather than decomposition of the vitamin.

The riboflavin (vitamin B_2) requirements of different species of animals under various nutritional or physiological conditions have been studied by many investigators and their results have been reviewed (1, 2). Although the nutritional requirements and biological functions of this vitamin have been well-established, the reason for the continuing need of riboflavin ingestion by animals is less well-known. Bessey et al. (3) attributed the rat "maintenance requirement" of riboflavin to loss through decomposition within the body and showed indirectly that a large quantity of riboflavin can be destroyed by the rat. Urinary excretion was thought to account for very little of the maintenance requirement, and the fecal riboflavin was believed to be not of dietary origin (3, 4). However, no degradative product has ever been found that resulted from the degradation of riboflavin in animal tissues. One group of workers has reported that riboflavin is not decomposed in the mammalian body (5).

Most of the previous studies were undertaken with either overdosage or deficiency of the vitamin. Difficulties were encountered because of the lack of sensitive methods for quantitative assay and for differentiating the ingested riboflavin from that synthesized by intestinal microorganisms.

In the present work these problems were overcome through the use of ^{14}C-riboflavin which can be accurately measured and is readily distinguishable from symbiotic riboflavin. It thus provides more valid data regarding degradation and the nature of the requirement of this vitamin under normal physiological dosage. Furthermore, the procedure for metabolic study is greatly simplified by the use of a radioactive tracer.

EXPERIMENTAL

Young male adult rats of the Sprague-Dawley strain,[3] weighing 170 g were used, and were maintained with commercial laboratory ration.[4] For some of the experiments, dietary riboflavin and succinylsulfathiazole were also included. 2-^{14}C-Riboflavin [5] was dissolved in 0.9% NaCl solution and 2 ml of the solution containing specified amounts of the riboflavin were injected intraperitoneally into each of 3 rats in each experiment. The rats were then kept in a metabolism cage, and the exhaled CO_2, urine, and feces were collected. At the end of the experiment the animals were decapitated and the urine, feces, and carcass were analyzed for the

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[2] Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, April, 1967.
[3] Blue Spruce Farms, Inc., Altamont, New York.
[4] Big Red Animal Food, Agway, Inc., Syracuse, New York.
[5] Nuclear-Chicago Corporation, Des Plaines, Illinois.

^{14}C compounds. All experiments were performed in the dark to avoid the photodecomposition of riboflavin.

Two types of metabolism cages were used in these studies. One was the commercially available open metabolism cage which can be used to collect urine and feces at any specific period of time. However, extensive washing is required to recover all the ^{14}C-compounds in urine because the urine tends to adsorb and dry on the collecting system. The other type was an all-glass closed metabolism cage made from an ordinary desiccator. With this type of cage, the exhaled $^{14}CO_2$ of the rats can be accurately measured, and generally a higher recovery of radioactivity from urine and feces was obtained. To assure normal physiological conditions, exhaled air was collected several times, for 3-hour periods, during the experiment. The total $^{14}CO_2$ exhalation was estimated by multiplying by the time factor.

Preparations of the samples. A modification of the method reported by Bessey et al. (3) was used. The urine was collected in a flask containing hydrochloric acid to prevent bacterial growth, and samples were made to a final concentration of 0.1 N in HCl. All solid samples were homogenized, added to 10 to 15 volumes of diluted hydrochloric acid (made to a final concentration of 0.1 N in HCl) and hydrolyzed by autoclaving 15 minutes at 120°. Unless otherwise stated the "carcass" refers to the whole body of the rat including the intestinal contents, which had been carefully extruded, followed by washing of the intestine. For the preparation of carcass samples, the rats were killed, chopped into pieces, and then frozen. The frozen carcass was ground with dry ice in a meat grinder, blended extensively in a Waring Blendor and hydrolyzed.

The samples prepared by the above methods are considered to be homogeneous hydrolysates in which ^{14}C-compounds are evenly distributed. The radioactivity of the sample was measured by counting aliquots of the sample and the ^{14}C-compounds were analyzed by the following procedures.

Analytical procedure. The general procedure for analysis is shown in figure 1. The hydrolysate was first extracted with an equal volume of water-saturated chloroform which removed some of the degrada-

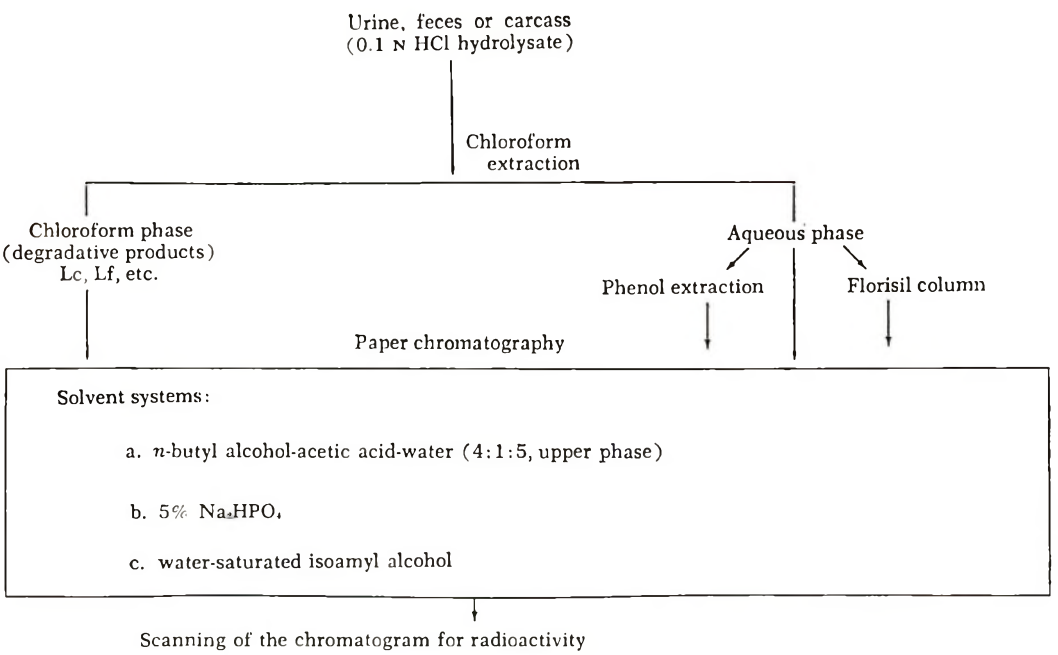

Fig. 1 Scheme of analytical procedure.

tive products such as lumichrome (6,7-dimethylalloxazine) and lumiflavin (6,7,9-trimethylisoalloxazine) from the aqueous phase. These samples were then analyzed by paper chromatography. In some cases the aqueous sample was spotted directly on paper, but better resolution was obtained when the sample was concentrated and cleaned by Yagi's "phenol extraction" procedure (6) or by a Florisil [6] column. In the latter procedure flavins were adsorbed by Florisil, and washed with 1 liter each of 5% acetic acid, distilled water and 0.5% pyridine. The flavin was eluted with 5% pyridine and the solution was taken to dryness.

Aliquots of the samples were spotted on Whatman no. 1 filter paper and were resolved by ascending chromatography in the following 3 solvent systems: 1) *n*-butyl alcohol-acetic acid-water (4:1:5, v/v/v, upper phase); 2) 5% Na_2HpO_4 aqueous solution; or 3) water-saturated isoamyl alcohol. The chromatogram was then scanned for radioactive spots with a radiochromatogram scanner [7] and compounds were identified by R_F values. The recovery of riboflavin from the sample was measured by cutting out the riboflavin spot and determining the radioactivity with a scintillation counter.

Assay of radioactivity. All measurements were made in a Packard Tri-Carb liquid scintillation spectrophotometer, with Bray's solution (7) as the liquid scintillator. Aliquots of 0.2 or 0.5 ml of aqueous sample in this scintillator gave satisfactory counting results, whereas for the samples in the chloroform or ether phases, aliquots (1 or 2 ml in each vial) were oven-dried before the addition of Bray's solution. In measuring the radioactivity of $^{14}CO_2$ which was absorbed into 4 or 8% NaOH solution, Bray's solution containing 4% thixotropic gel was used as the liquid scintillator to prevent precipitation of the sample in the counting vial. In all cases, ^{14}C-benzoic acid was used as an internal standard to determine the counting efficiency of each vial and all data were expressed as disintegrations per minute.

Depletion of ^{14}C-riboflavin from the rat. After the administration of ^{14}C-riboflavin, the rats were kept in a metabolism cage. Urine and feces were collected every 3 hours early in the experiment and at longer intervals later. At the end of the experiment, almost all radioactivity administered could be recovered from the samples of urine, feces, and carcass. Therefore, the body content of ^{14}C-riboflavin at a specified time was calculated by subtracting the amount of radioactivity excreted from that initially administered. A semilogarithmic plot of body content versus time was used to estimate the rate of depletion of the vitamin from the animal.

Experiments in vitro. 2-^{14}C-Riboflavin with a final concentration of 3×10^{-6} M was incubated with homogenates of rat liver, kidney, muscle, intestines, or the contents of the cecum and large intestine at 35° for 2 or 3 hours. The reaction mixture was buffered with 7×10^{-2} M sodium phosphate solution (pH 7). Heat-inactivated homogenates were incubated in the same way as the control. For the detection of $^{14}CO_2$ production during incubation, the reaction mixture was incubated in a 50-ml Erlenmeyer flask stoppered with a rubber septum. The reaction was terminated by an injection of 20% trichloroacetic acid and the $^{14}CO_2$ was collected into a solution of hydroxide of Hyamine [8] adsorbed on a filter paper wick in a polypropylene center well appended to the rubber septum. The radioactivity of the $^{14}CO_2$ collecting unit was then measured in a liquid scintillation counter. The incubated mixture was extracted with an equal volume of water-saturated chloroform. Radioactive compounds extracted into the chloroform were counted and examined by paper chromatography.

RESULTS

Distribution of radioactivity after administration of ^{14}C-riboflavin. The distribution of radioactivity at 24 hours after administration of 2-^{14}C-riboflavin is shown in table 1. Approximately 13% of the radioactivity was excreted through urine and feces. About 81% was retained in the body, most of it being in the liver and

[6] Florisil (60–100 mesh), Floridin Company, Tallahassee, Florida.
[7] Actigraph III, Nuclear-Chicago Corporation.
[8] Hydroxide of Hyamine 10-X, Packard Instrument Company, Inc., Downers Grove, Illinois.

"rest of the carcass." On the basis of disintegrations per minute per gram fresh weight of tissue, the values are high in liver and kidney, being 375,000 and 261,000 dmp/g, respectively, as compared with the 67,400 dmp/g administered. The values in the intestines were at about the same level as those administered, whereas the value for the "rest of carcass" was only half that of the administered level, and the radioactivity in the contents of intestines was low. The radioactivity was extremely low in the blood; therefore, the blood was not analyzed in later experiments. The chloroform-soluble compound(s) (CSC) were very low in all samples except those of feces and the contents of the cecum and large intestine.

Recovery of radioactivity. Table 2 presents the results of four separate experiments. In all cases, relatively high recoveries of radioactivity (approximately 95%) were obtained. The distribution patterns of ^{14}C-compounds in urine, feces, and carcass are related to the body size of the animal as well as to the amount of riboflavin administered. Generally, higher body retentions of ^{14}C-compound were observed for experiments with larger animals, and with lower levels of ^{14}C-riboflavin administration. Exhaled CO_2 was collected in experiments 3 and 4. Six random collections, each of 3-hour duration, were made during each of the 2 experiments. The production of $^{14}CO_2$ was detectable in both experiments, but the quantity was

TABLE 1

Distribution of radioactivity 24 hours after administration of ^{14}C-riboflavin [1]

	^{14}C-riboflavin		CSC [2]
	%	*dpm* × 10^3/*g* [3]	%
Liver	27.86	375	0.24
Kidney	4.32	261	0.36
Small intestine (minus contents)	4.57	84	0.21
Contents of small intestine	1.50	30	0.29
Cecum and large intestine	1.82	63	0.25
Contents of cecum and large intestine	0.49	4.2	1.40
Blood	0.02	—	—
Rest of carcass	40.30	33.6	0.28
Urine	10.17	—	0.68
Feces	2.89	—	3.23
Total	93.89		

[1] Each of 3 rats weighing 170 g was injected with 55 μg (5 μCi) of 2-^{14}C-riboflavin.
[2] Chloroform-soluble compound(s) — % radioactivity that can be extracted into the chloroform phase from the aqueous sample (see analytical procedure).
[3] Radioactivity administered 67.4 × 10^3 dpm/g rat.

TABLE 2

Recovery of radioactivity after administration of 2-^{14}C-riboflavin

Experiment no.	1		2		3		4	
(No.) and avg wt of animals, g	(3) 110		(3) 170		(3) 104		(2) 165	
^{14}C-riboflavin administered, μg	80 (5 μCi)		55 (5 μCi)		55 (5 μCi)		83 (7.5 μCi)	
Length of experiment, hr	24		24		72		100	
	%	% CSC	%	% CSC	%	% CSC	%	% CSC
Recovery in								
Urine	26.2	0.23	10.2	0.68	19.2	0.43	24.6	0.61
Feces	5.8	5.50	2.9	3.23	12.8	1.72	20.4	2.27
Carcass	62.4	0.12	80.8	0.31	65.6	0.26	50.5	0.09
$^{14}CO_2$					tr		tr	
Total	94.4		93.9		97.6+		95.5+	
^{14}C-riboflavin solution		0.11		0.43		0.48		0.31

very low and was much less than 1% of the ^{14}C administered.

The % CSC values range 2 to 5% for feces, whereas for urinary and carcass samples, the values are in the same range as that for the ^{14}C-riboflavin solution. In the aqueous phases of the samples of urine, feces, and carcass, no degradative product was detected as judged by paper chromatography in the three solvent systems used. However, the presence of trace amounts of degradative products in the samples has not been ruled out. Complete recovery of radioactivity from the riboflavin spot on the chromatogram was not obtained. Generally, 70 to 95% of the radioactivity applied could be recovered from the riboflavin spot, and usually the recovery was lower for fecal than urinary samples.

The nature of the chloroform-soluble compounds in feces. The dietary effects of the % CSC are shown in table 3. For the rats that had been maintained with the riboflavin-supplemented diet for 2 weeks, the % CSC in feces was found to be 10% as compared with a value of 3% for rats fed a laboratory ration. On the other hand, for the rats fed a riboflavin-supplemented diet for the first week and an additional 1% succinylsulfathiazole for the second week, the % CSC was reduced to 4%. A similar relationship was also observed for the contents of cecum and large intestine. The % CSC values of other samples are very low and are in the same range as those for the ^{14}C-riboflavin solution. As shown in tables 1 and 3, in all cases the % CSC values in the contents of the cecum and large intestine are higher than those of the cecal and large intestinal tissues as well as all other samples. It appears that the site of degradation is in the contents of the cecum and large intestine rather than in the tissues of the rat.

The chloroform-soluble compound was identified as lumichrome, as judged by its chromatographic behavior in three different solvent systems, and by its fluorescence spectra after purification through an alumina column. The fluorescence spectra are shown in figure 2 together with those of the photochemically produced lumichrome. In the experiment with 500 μg of ^{14}C-riboflavin administered to rats maintained with the high riboflavin diet, the % CSC value was found to be as high as 20%. Both lumichrome and lumiflavin (which appeared in a 7:3 ratio) were identified as degradative products, as judged by paper chromatography and by fluorescence spectra. Some of the R_F values of lumichrome, lumiflavin, riboflavin and riboflavin-5′-phosphate (FMN) are shown in table 4.

In the experiments in vitro, $^{14}CO_2$ or ^{14}C-lumichrome, or both, were detected as degradative products when ^{14}C-riboflavin solution was incubated with the contents of cecum and large intestine. As the production of these compounds was very low,

TABLE 3

Effect of riboflavin-enriched diet and succinylsulfathiazole on percentage of chloroform-soluble compound(s) (CSC) [1]

Diet	Laboratory ration	Laboratory ration + riboflavin (15 μg/g diet)	Laboratory ration + riboflavin + 1% succinylsulfathiazole
(No.) and avg wt of animals, g	(2) 110	(3) 110	(3) 110
^{14}C-riboflavin administered, μg	110 (10 μCi)	110 (10 μCi)	110 (10 μCi)
	% CSC	% CSC	% CSC
Recovery in			
Urine	0.25	0.32	0.21
Feces	3.10	9.91	4.45
Cecum and large intestine (minus contents)	0.25	0.39	0.20
Contents of cecum and large intestine	1.50	3.20	1.36
Rest of carcass	0.36	0.56	0.46
^{14}C-riboflavin solution	0.55	0.55	0.55

[1] Each experiment was carried out for 50 hours.

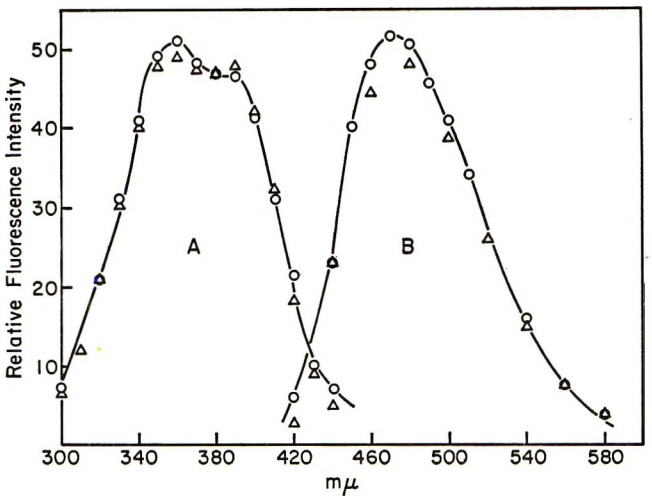

Fig. 2 Flourescence spectra of the fecal lumichrome in water; *A*, wavelength of excitation when measuring fluorescence at 480 mμ; *B*, wavelength of fluorescence with excitation at 360 mμ. Symbols: ○, lumichrome isolated from feces; △, photochemically produced lumichrome.

TABLE 4

R_f values of riboflavin metabolites

Solvent system	Lumichrome	Lumiflavin	Riboflavin	FMN
Butanol-acetic acid-water	0.62	0.40	0.28	0.05
5% Na_2HPO_4	0.05	0.10	0.26	0.50
Water-saturated isoamyl alcohol	0.44	0.20	low	low

quantitative data were not obtained. On the other hand, no degradative product was detected when ^{14}C-riboflavin was incubated with the homogenates of rat liver, kidney, muscle or intestinal tissues.

The depletion of ^{14}C-riboflavin. The excretion pattern of ^{14}C-riboflavin during the first 48 hours after administration is shown in figure 3. Urinary excretion of the ^{14}C-compound was rapid during the first few hours after administration, whereas, there was a latent period of several hours before fecal excretion of the compound was noted. After 10 hours, both urinary and fecal losses increased steadily, though not at the same rate.

The depletion of ^{14}C-riboflavin during the first 10 days of the experiment is shown in figure 4. After the first 24 hours, both urinary and fecal excretions followed approximately a course of exponential removal. The average daily rate of excretion

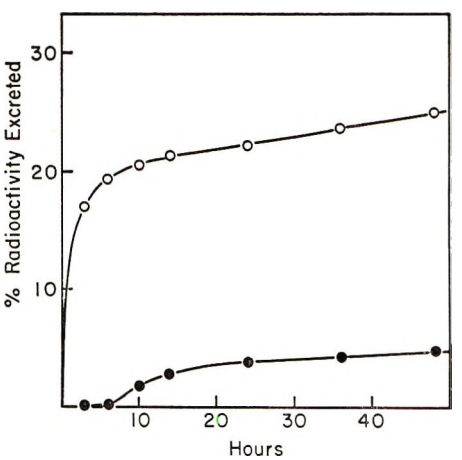

Fig. 3 The excretion of ^{14}C-riboflavin during the first 48 hours after administration. Fifty-five micrograms (5 μCi) of 2-^{14}C-riboflavin were administered to each of 3 rats weighing 100 g. Symbols: ○, accumulative urinary excretion; ●, accumulative fecal excretion.

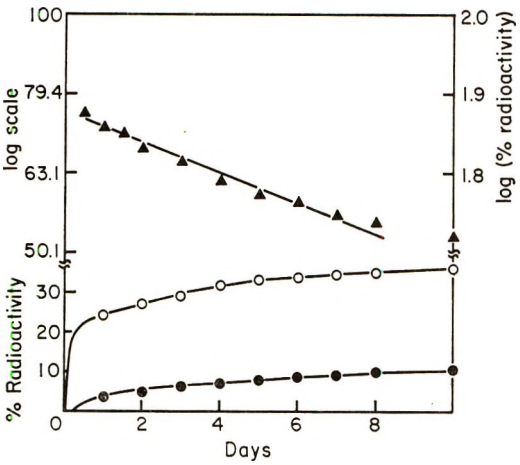

Fig. 4 Depletion of ^{14}C-riboflavin in the first 10 days after administration. Symbols: ○, urinary excretion; ●, fecal excretion; ▲, calculated body retention. The data are averages of 2 experiments carried out under the conditions described in figure 3.

during the second to eighth days was estimated to be 1.6% of the radioactivity administered for urinary and 1.0% for fecal excretion. On plotting the body retention in a logarithmic scale versus time, the best-fit straight line for the data during days 1 to 7 was found to represent a depletion rate of 0.043 per day. This indicates also that the tissue riboflavin has a half-life of 16 days in rats under normal physiological conditions and fed a laboratory ration.

Upon examination of the nature of the ^{14}C-compounds in urine and feces, results similar to those in experiments 1 to 4 (table 2) were obtained for both urinary and fecal samples for the first 3 days of the experiment. In the urine samples of the last days of the experiment, approximately 70% of the ^{14}C-compound was recovered as ^{14}C-riboflavin on paper chromatograms; but no spot of degradative product was detected. The radioactivity of the fecal samples in the latter days of the experiment was very low and satisfactory quantitative recovery of riboflavin was not obtained. However, at least half of the radioactivity in these fecal samples is estimated to be due to intact riboflavin.

The % CSC in feces was lowered to 1% in the last days as compared with 4 or 5% for the earlier days of the experiment. Change of chloroform extraction pattern was not observed for the urine samples. Upon extracting the samples with equal volumes of water-saturated phenol, the fraction of radioactivity remaining in the aqueous phase was found to increase with time for both urinary and fecal samples, from 4 or 5% on day 1 to about 15% on day 8. Values for the standard ^{14}C-riboflavin solution and the carcass were from 2 to 3%. Due to the low radioactivity of these samples, the nature of the ^{14}C-compounds remaining in the aqueous phase was not determined. However, the extraction pattern obtained may give an indication of the breakdown of the isoalloxazine ring structure during the experiment.

DISCUSSION

The patterns of ^{14}C-riboflavin retention and excretion in all the experiments are consistent with the finding that there are rather well-defined maxima and minima for tissue riboflavin concentrations (3). There was a rapid urinary loss of ^{14}C-riboflavin during the first few hours after administration. The fraction of radioactivity excretion was proportional to the amount of riboflavin administered and inversely proportional to the body size of the animals.

The data in table 1 represent the general patterns of riboflavin incorporation into the tissues of the animal. The high value of the disintegrations per minute per gram for kidney may be attributed partially to its functions, namely, the excretion and reabsorption of the vitamin (8, 9). Yagi and co-workers (5) have reported a much higher value of radioactivity in the kidney as well as in the small intestine at one hour after subcutaneous injection of the rat with ^{14}C-riboflavin. In view of the large amount of riboflavin injected (about 60 times the daily requirement), their data appear to provide information regarding the migration and excretion of riboflavin rather than the incorporation and distribution of this vitamin in the animal.

The high recovery of radioactivity and the inefficient production of $^{14}CO_2$ observed in the present studies are in good agreement with the results reported by Faulkner and Lambooy (4). They observed a 98% total recovery of radioactivity from urine, feces, and carcass after maintaining rats

with a riboflavin-deficient diet for 4 weeks. The $^{14}CO_2$ and other gas losses of radioactivity, therefore, are not quantitatively significant and almost all ^{14}C-compounds would be expected to remain in the urine, feces, and carcass. Aside from the chloroform-soluble lumichrome and lumiflavin in the feces, no degradative product of riboflavin was detected by paper chromatography. Although there were indications of decomposition of the isoalloxazine nucleus in the urinary and fecal samples in the latter days of the experiment, the recovery of ^{14}C-riboflavin from the samples was still high. The relatively large amount of riboflavin excretion suggests that the ability of tissues to decompose this vitamin is relatively low. This is supported by the experiments in vitro and by the fact that no tissue degradative product of riboflavin has ever been identified. Owen and coworkers (10, 11) have isolated 6, 7-dimethyl-9-(2′-hydroxyethyl)isoalloxazine which occurs in a few parts per million of goat urine as a riboflavin metabolite, but in the later reports of these workers,[9,10] they tended to believe that it is a symbiotic product.

Bessey et al. (3) have reported a total recovery of 25% at 24 hours after administering 500 μg of riboflavin to a rat that had been maintained with a high riboflavin diet and claimed that the remaining 75% of riboflavin was destroyed in the rat. When we attempted to repeat this experiment, 52% of the radioactivity administered was excreted in urine during the first 24 hours of the experiment and no degradative product was detected in the urinary sample. This result, as well as the other observations of the present studies, suggests that the overall 25% riboflavin recovery is too low and that there was an over-estimation of the riboflavin-decomposing ability of the rat by Bessey et al. (3).

It is well-known that microbial enzyme activity can be induced by an enriched medium, and that sulfa drugs are antibacterial substances. The dietary effects on the level of % CSC in feces (table 3), as well as other experiments in vivo and in vitro, indicate that the riboflavin degradative products, CO_2, luminchrome, and lumiflavin are produced by the intestinal microorganisms. Lumichrome is a well-known degradative product of flavins and the production of lumiflavin has also been reported (12, 13).[11] There is also evidence that the ribitol side chain of riboflavin is not degraded by rat tissue under normal physiological conditions.

The body retention data in figure 4 do not fit exactly into a straight line. They tend to curve up, especially for the data of the latter days, due partially to growth of the rats during the period of the experiment. The half-life of 16 days for tissue riboflavin is in good agreement with that reported by Amos and co-workers.[12] The turnover rate of this vitamin in rats is directly affected by the level of the dietary riboflavin. A much slower turnover rate has been reported for rats fed a riboflavin-deficient diet (4) and a much shorter half-life has been observed for rats fed a high riboflavin diet.[13] Assuming a body concentration of 400 μg of riboflavin for a 100-g rat (3), the observed depletion rate of 0.043 corresponds to an excretion rate of 17.2 μg of riboflavin daily. This amount is very close to the value of 16 μg of riboflavin which is required to maintain tissues (maintenance requirement) of a corresponding growing rat (3) and is 12 times higher than the depletion rate as reported for riboflavin-deficient rats (4). In contrast with the previous report that there is little or no riboflavin excretion into urine and feces (3), the present studies show that most of the radioactivity excreted by the animal is that from ^{14}C-riboflavin. Thus, the loss of riboflavin from a growing rat, under normal physiological conditions, is due mainly to excretion rather than to decomposition of this vitamin.

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their help in the preparation of this manuscript.

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Intestinal Absorption and Body Retention of ^{47}Ca in Rats: Evaluation of the method [1]

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ABSTRACT A method is described to study intestinal absorption and body retention of calcium in rats which requires the use of oral ^{47}Ca and monitoring of body radioactivity by a whole body counter. Extrapolation to zero time of the monoexponential portion of the body retention curve of ^{47}Ca was used to estimate net intestinal absorption. The slope of this curve provided an index of body retention of calcium. The validity of the estimate of intestinal absorption was confirmed by direct analysis of fecal, intestinal tract and carcass content of ^{47}Ca after oral administration. Quantitative data on intestinal absorption and body retention of ^{47}Ca after oral dose were obtained in various groups of normal rats of increasing weight and age. Growth and maturation had a depressing effect on both intestinal absorption and whole body retention of ^{47}Ca.

Several methods have been used to study the intestinal absorption and body retention of calcium in the rat. The conventional balance method has been used by many investigators (1–4). By this technique, apparent absorption was derived from subtraction of fecal calcium from ingested calcium, and body retention (or balance) was calculated from the difference between intake and the combined urinary and fecal losses of calcium. With the increased availability of radioisotopic calcium, it became clear that the estimate of intestinal absorption obtained by the balance method is inaccurate. Thus, Hansard and Crowder (5), using a combined balance and radicisotopic method in rats, showed that with age substantial and increasing amounts of endogenous calcium are excreted in the feces, and that the observed apparent absorption, determined by simple balance, should be credited with this amount of fecal endogenous calcium.

Methods utilizing labeled calcium are varied and numerous. Wasserman and Comar (6) used ^{45}Ca uptake of the femurs, 24 hours after an oral dose, as a measure of gastrointestinal absorption. In short-term (7-hour) experiments with rats, Marcus and Lengemann (7) calculated absorption from the radioactivity recovered in the intestinal segments at different intervals after an oral dose of ^{45}Ca. Using more direct procedure of counting carcass ^{45}Ca 7 hours after oral dose, Taylor et al. (8) estimated absorption by subtracting gastrointestinal contents from combined whole body and urinary ^{45}Ca. The combined balance and labeled calcium method of Hansard and Crowder (5) and the similar method of Aubert et al. (9) appear to be the most accurate and complete.

Methods which require direct assay of ^{45}Ca content of tissue or excreta are time-consuming, and the coincident balance method requires constant experimental conditions of sampling over protracted periods. The availability of ^{47}Ca, a gamma-emitting radioisotope of calcium, and the use of a whole-body counter have made it possible to utilize external counting in serial study of intestinal absorption and whole body retention of rats under varying physiologic conditions. The present report examines such a technique. Rapid computation of both processes was derived from analysis of the whole-body radioactivity curve; the method was found to be consistent with direct determination of carcass and intestinal tract radioactivities.

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EXPERIMENTAL

Material. Male rats of the Sprague-Dawley strain were used in all the studies. Animals were usually acquired when three to four weeks old, weighing between 45 and 60 g, and were weighed twice weekly to provide an estimation of growth rate and general physical condition. Rats showing the best growth rates were selected for experiments and transferred to separate mesh-bottom cages to provide ample space for each and to minimize coprophagy. In appropriate instances, metabolic cages were used to facilitate accurate collections of feces and urine. The animals were allowed free access to food and water. Two commercial laboratory feeds were utilized over the course of these studies, one [2] containing 1.42% Ca, 0.96% P, and the other [3] containing 0.78% Ca, 0.73% P. The former was used in most experiments. While both diets are similar in protein content, sustained optimal growth, and were relatively high in calcium content, the Ca:P ratio was 40% lower in the latter diet and may have introduced significant differences in experiments repeated in the same age animals one year apart. For this reason, the data presented are selected so that each animal group served as its own control and received the same diet throughout the entire experimental series. The approximate chemical composition of the 2 diets used is presented in table 1. Each experiment was conducted with sufficient numbers of rats to facilitate statistical computation and practical accuracy.

Method. $^{47}CaCl_2$ (mean specific activity: 165 μCi/mg Ca) was obtained monthly from Oak Ridge. The rats were fasted overnight, and then fed by gavage in the morning under light ether anesthesia. One-half to one microcurie of ^{47}Ca ($CaCl_2$) in one milliliter of non-fat milk was given to each rat (stable calcium: 0.8 mg). Three hours after gavage feeding the animals were again allowed free access to food and water. To determine whole-body radioactivity the rats were placed in cylindrical cartons appropriate to body size and counted for one minute or more; ^{47}Ca was measured in a total body scintillation counter,[4] connected with a scintillation spectrometer [5] with the channel set at the 1.31-mev peak for the gamma-radiation from ^{47}Ca. The lower radiation energy (0.16 mev) of the immediate decay product, scandium-47, could be eliminated by this procedure.

TABLE 1

Approximate chemical composition of diets used in experimental series [1]

Diet	A [2]	B [3]
Protein, %	23.40	20.50
Ca, %	1.42	0.78
P, %	0.96	0.78
Ca:P	1.48	1.00
Total, %	3.80	10.50
Vitamin D, IU/g	5.31	3.40

[1] The change in diet was recognized only in retrospect, and was due to a change in the institutional contractor supplying the stock diet routinely fed to the rat colonies.
[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[3] Wayne Lab Blox, Allied Mills, Inc., Chicago.

Total body counts were performed within one hour after gavage feeding to determine the dose administered, and repeated at appropriate intervals thereafter (for example, 1, 2, 3, 5, 8 and 12 days). All counts, corrected for background and physical decay, were expressed as percentage of the administered dose (initial body radioactivity). The percentage of retained ^{47}Ca in each rat was plotted on semi-log paper against time (fig. 1). The curve so obtained fell rapidly during the first 3 days, then appeared to fall linearly thereafter.

Extrapolation of the straight portion of the curve back to zero time was used to estimate the *net intestinal absorption* (A). The measurement of the slope (B-C) of this portion of the plot of retained ^{47}Ca permitted derivation of the rate of body loss of absorbed ^{47}Ca. It was expressed in days half-life ($T_{1/2}$), and as a constant *K*. *K* represents the daily percentage loss of calcium from the whole-body exchangeable calcium mass and is derived from the standard formula, $K = 0.693 \times 100/T_{1/2}$. These values were termed *body retention.*

Validation experiments. ^{47}Ca content of intestinal tract, feces, urine and carcass was directly measured at several intervals after gavage feeding and compared with whole-body counts. Young male rats of the same origin and age, weighing 80 to

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis.
[3] Wayne Lab Blox, Allied Mills, Inc., Chicago.
[4] Packard Model 445 Armac Scintillation Detector.
[5] Series 314-E, Packard Instrument Company, Inc.

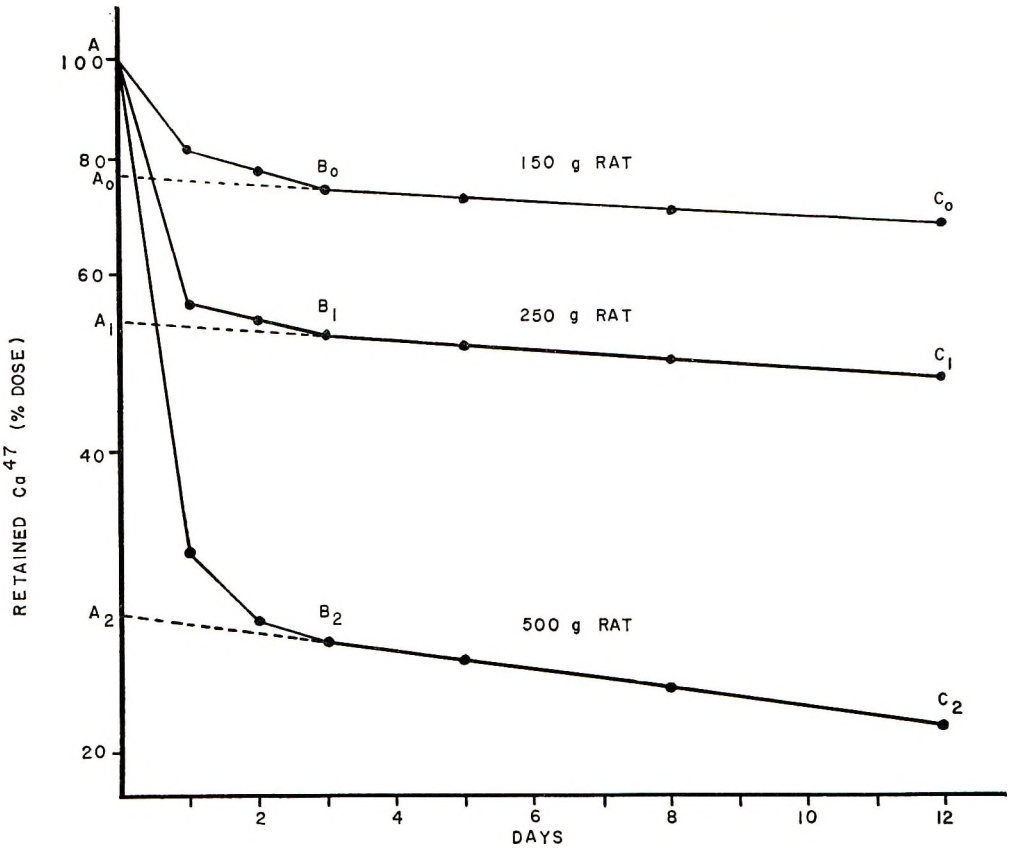

Fig. 1 Whole body radioactivity after gavage feeding of ^{47}Ca. All counts were arbitrarily assigned 100% at time zero (A). The back extrapolated to time zero is shown as the dotted line to A_0, A_1 and A_2. The plot appears linear after the third day.

100 g, were chosen for this study on the basis of their nearly identical high intestinal absorption and body retention of ^{47}Ca. A control, non-killed group was counted in the whole-body counter for 12 days, as in the standard procedure.

In this experiment the amount of ^{47}Ca fed was also assessed by recording ^{47}Ca content of the syringe and tubing before and after gavage feeding. This equipment, as well as the intestinal, fecal and urine specimens were counted in glass test tubes which were inserted horizontally into the center of a carton of the same type used for total-body counting. By keeping a consistent geometry, the respective radioactivities of these specimens could be expressed in a manner equivalent to that of the carcass and whole body.

Groups of 4 rats were killed at 3, 5, 7, 12, 24 and 48 hours. Total-body radioactivity was measured before dissection, then the abdominal cavity was opened and the cardia of the stomach and the ileocecal junction were immediately clamped. The intestinal tract was removed from (and including) the cardia to the rectum, and divided into 2 parts (1 and 2, table 2) at the ileocecal junction. These tissues were placed in separate test tubes and counted as described above. Feces and urine obtained prior to killing were counted in the same manner. Finally, the ^{47}Ca content of the carcass was measured. Data were expressed as percentage of initial dose of ^{47}Ca, and as percentage of postmortem total body ^{47}Ca.

TABLE 2

Intestinal absorption of ^{47}Ca studied at intervals by direct measurements of carcass and intestinal tract radioactivities in 100-g normal rats

Hours after oral dose rats were killed	Whole-body counting method [1]		Test tube counting method				
	At time of killing	In carcass	Urine	Intestinal tract [1]			Absorption [2]
				Feces	1	2	
	% whole-body radioactivity						
3	100.0	88.9	0	0	5.5	5.2	89.3
5	100.0	94.4	0	0	0.9	5.0	94.1
7	100.0	94.3	tr [3]	0	1.4	3.7	94.9
12	98.6	92.6(91.3) [4]	tr [3]	1.4	0.8	7.1	90.7
24	94.3	92.8(98.4)	tr [3]	7.4	1.2		91.4
48	93.9	93.9(100.0)	tr [3]	5.1	0.3		94.6

[1] Average values expressed as percentage of initial dose.
[2] Compared with the 94.0% value of absorption obtained by the total-body counting method in controls (not killed) (12 days), utilizing extrapolation.
[3] Less than 0.001% of dose.
[4] In parentheses the values are expressed as percentage of total-body ^{47}Ca at time of killing.

RESULTS AND DISCUSSION

Whole-body radioactivity after oral administration of ^{47}Ca. After a first phase of rapid fall, lasting 2 to 3 days, the curve of whole-body radioactivity appeared to follow a slow single exponential decline through day 12 (fig. 1). The initial decrease in whole-body radioactivity was smallest in young rats (AB_0 and AB_1), and greatest in the older animals (AB_2). Determinations of corresponding fecal and urinary ^{47}Ca indicated that the loss in body ^{47}Ca was almost entirely due to fecal ^{47}Ca. If it is assumed that the gut was cleared of unabsorbed ^{47}Ca at the end of the first phase (points B_0, B_1, B_2), the whole-body content of ^{47}Ca thereafter must represent the absorbed ^{47}Ca, and the subsequent monoexponential fall must then result from that portion of absorbed ^{47}Ca which is subsequently secreted into the intestinal juices and excreted in the feces.

Extrapolation of the straight portion of the retention curve back to zero time (points A_0, A_1, A_2) would then indicate the percentage dose retained (or percentage dose absorbed) if no excretion of absorbed ^{47}Ca occurred. This value was thereafter assumed to estimate the net intestinal absorption. The slope of the straight portion of the body retention curve reflects the rate of body loss of absorbed ^{47}Ca. The younger animals exhibited the lowest rate of body loss of ^{47}Ca (slow decline B_0, C_0 and B_1, C_1), as compared with the higher loss in the older rats (steeper slope B_2, C_2). The loss of ^{47}Ca from the body is the resultant of 1) the intestinal secretion of ^{47}Ca previously absorbed, less 2) the reabsorption of ^{47}Ca from the bowel lumen. Ionic exchange of ^{47}Ca in the bowel lumen with ^{40}Ca in extracellular fluid may also be a factor, but probably unimportant in magnitude as it appears likely that in the mating rats the bulk of calcium absorption takes place in a proximal region of the bowel, and distal secretion of calcium is largely unopposed. As such, ^{47}Ca is excreted into the feces (intially along with the fed ^{47}Ca which escaped absorption). This loss of ^{47}Ca is reflected in the slope of body loss, the whole-body retention. If, however, significant distal absorption of calcium occurs, as is the case in young rats (see later), then the magnitude of the absorptive process in the large bowel may importantly affect the body retention curve (10).

Validation of the method of external counting for measurement of intestinal absorption and body retention of calcium. The assumption that intestinal absorption and body retention of ^{47}Ca could be calculated from the curve of whole-body radioactivity implies that after some time, presumably after intestinal clearance of nonabsorbed calcium, the recorded whole-body radioactivity corresponds to that present in the carcass, and thus to that absorbed. We, therefore, sought to find out by direct means how long and to what extent orally given ^{47}Ca was pooled in the intestinal tract.

The ^{47}Ca content of intestinal tract, feces, urine, carcass and whole body, at several intervals after gavage feeding, is presented

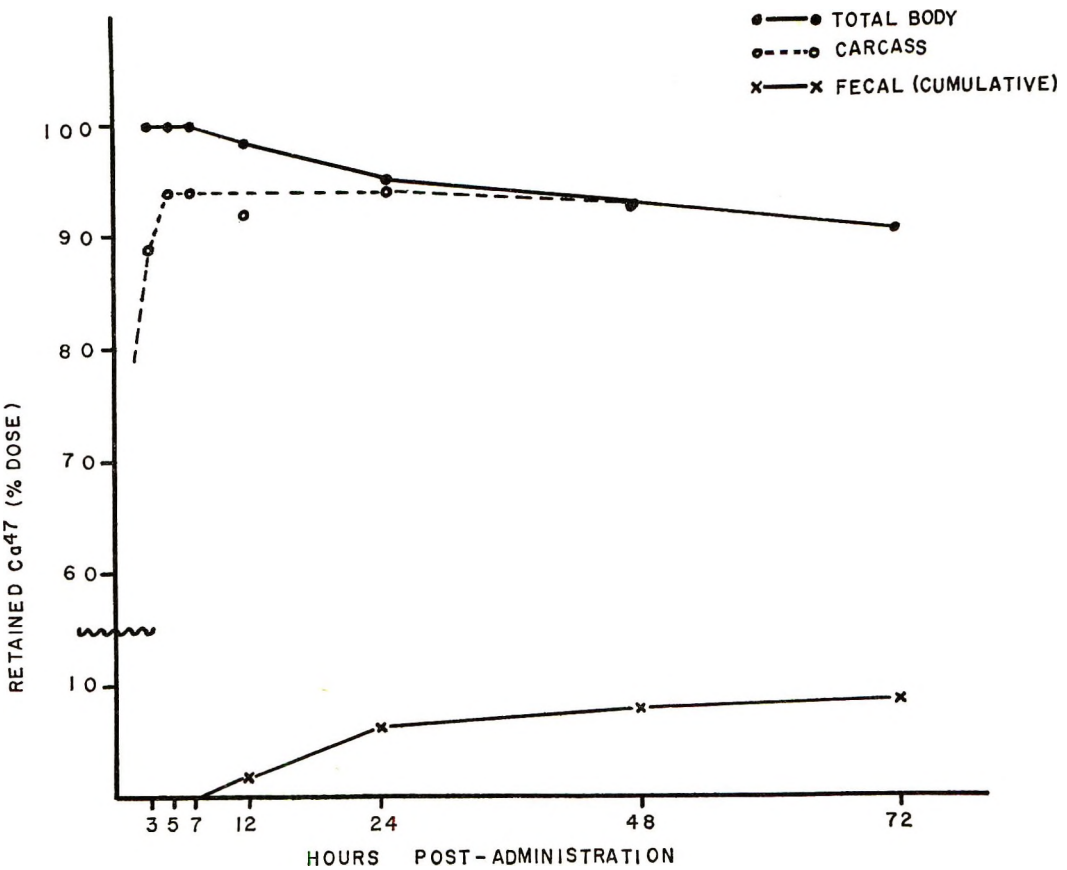

Fig. 2 ^{47}Ca content of feces, carcass and whole body. In the rat, virtually all losses of calcium are in the feces. Carcass radioactivity approximated whole body radioactivity at 24 hours.

in table 2 and figure 2. The curve of whole body ^{47}Ca of the non-killed controls is presented in figure 3. The results of this study indicated the following.

(a) Whole-body radioactivity did not change until 7 hours after gavage feeding, and then decreased gradually. This loss in whole-body ^{47}Ca was substantially due to fecal excretion of ^{47}Ca. Only traces of labeled calcium were found in the urine.

(b) The carcass radioactivity increased very rapidly to reach 88.9% of whole-body content of ^{47}Ca at 3 hours and 94.4% at 5 hours. Although the 12-hour group of rats showed a mean carcass radioactivity less than whole-body content, two of these rats had already cleared all intestinal radioactivity and carcass radioactivity approximated whole-body radioactivity. Practically all the whole-body radioactivity recorded after 24 hours was localized in the carcass.

(c) The amount of ^{47}Ca absorbed from the intestinal tract, as directly determined by carcass ^{47}Ca, was similar to that calculated by subtracting from the oral dose the directly measured intestinal ^{47}Ca. Furthermore, the mean absorption determined directly at 24 and 48 hours in the animals that were killed and the mean absorption of the controls not killed, calculated by the extrapolate method were virtually identical (94%). Thus, direct method of measurement of net intestinal absorption confirmed the validity of the total-body radioactivity method of extrapolation from the estimation of calcium absorption from the gut.

(d) After 24 to 48 hours, carcass radioactivity was identical with whole-body

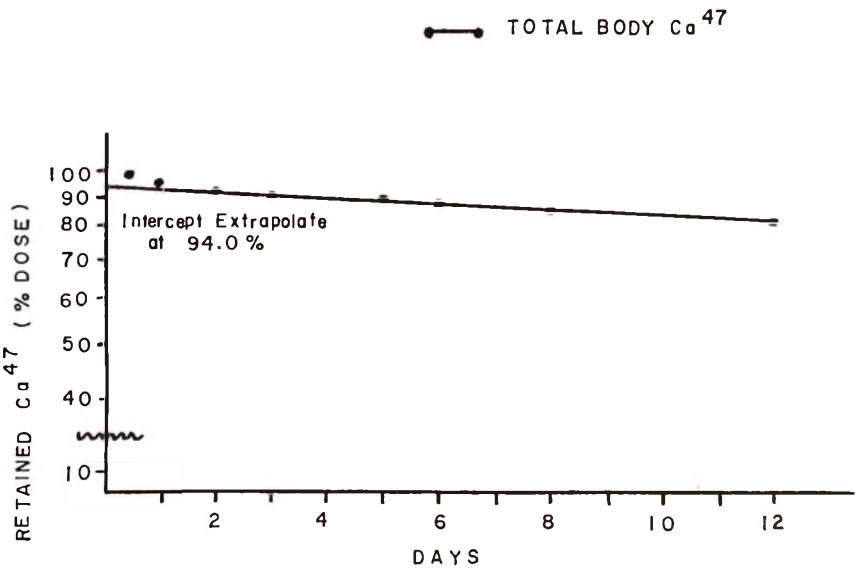

Fig. 3 Whole body content in non-killed controls. Compare 94.0% extrapolate with values in carcass in figure 2 (see text).

radioactivity. The decrease of whole-body ^{47}Ca after 48 hours reflected the loss of ^{47}Ca from the carcass. This rate of fall was monoexponential, and the slope reflects body retention of ^{47}Ca.

It can be assumed that during the period of monoexponential fall 1) most of the ^{47}Ca of the carcass was located in the bone, and 2) the endogenous ^{47}Ca secreted into the gut with the intestinal juices was derived from some rapidly exchangeable calcium pool (identified by Aubert-Milhaud (9) as Pool P) in equilibrium with the ^{47}Ca in the skeleton. Whether the loss of ^{47}Ca from bone was due in part to displacement of ^{47}Ca by subsequently absorbed stable calcium (nonlabeled), or was solely a reflection of the rate of bone resorption, could not be answered by these experiments.

This experiment appears to confirm previous reports that rats completely absorb calcium and strontium within 5 to 7 hours after oral administration (7, 8, 11). However, this was not always the case. In a similar experiment performed with 100- to 120-g young male rats in which absorption of oral ^{47}Ca by the extrapolate method was approximately 85 to 90%, carcass radioactivity was found to be only 50% of the administered dose of ^{47}Ca at 7 hours, and the rest was still pooled in cecum and colon. Evidence that, at least in young rats, this calcium may still be absorbed was obtained by demonstrating that up to 50% of ^{47}Ca injected directly into the cecum may be recovered in the carcass.

Application. Data on intestinal absorption and whole-body retention after gavage feeding of ^{47}Ca were obtained in *various* groups of normal male rats of increasing weights and age (table 3). The highest absorption of 87.6% was found in the youngest animals (100-g). There was a gradual fall with increasing age and weight. Absorption decreased rapidly after the rats reached weights of about 300 g. The same absorption was found in both 300- and 400-g groups, presumably because of their identical rate of growth. In the group of 1-year-old rats (weight 500 g), mean absorption was as low as 25%.

Whole-body retention of ^{47}Ca appeared to decrease with advancing maturation and age. The average K value of a group of 100-g young rats was 1.016, as compared with 2.484 of the 500-g group of rats.

These data indicated that growth and maturation have a depressing effect on the intestinal absorption and body retention of absorbed calcium in rats. It is likely that a combination of physiological proc-

TABLE 3

Intestinal absorption and total body retention of ^{47}Ca in normal rats (different animals)

No. of animals	Weight	Absorption of ^{47}Ca	Body retention of ^{47}Ca	
			$T_{1/2}$	K value
	g	*% of oral dose*	*days*	*% body loss/day*
16	100	87.6 ± 6.4 [1]	68.2 ± 24.4	1.016
18	200	74.8 ± 7.0	78.4 ± 7.0	0.884
10	300	36.5 ± 13.0	55.6 ± 14.6	1.246
18	400	35.5 ± 12.3	60.2 ± 20.3	1.151
12	500	25.0 ± 4.4	27.9 ± 6.3	2.484

[1] Mean ± SD.

esses regulate the rate of loss of body calcium. With age, more of the ^{47}Ca initially deposited in bone becomes available for intestinal secretion and once secreted into the gut with the intestinal juices, more of this endogenous calcium is lost in the feces (5). Whether the mature rat absorbs less calcium because of a lessened need, or whether the intestinal mucosal cells have a diminished ability to transport calcium, cannot be answered in these experiments.

The quantitative aspects of this study on intestinal absorption have to be considered cautiously within the purposely chosen limits of the experimental method, that is, diet was relatively high in calcium, calcium was administered as chloride in skim milk, the animals were fasted overnight, the gavage was performed in the morning, and so on. However, within these restrictions, it was found that the method offers a useful index of the efficiency of calcium absorption and permits objective comparisons when diseased rats are studied under identical conditions of diet and procedure.

The net absorption of ^{47}Ca after oral feeding reflects the *absorptive* efficiency of the entire intestinal tract for calcium of both the test dose administered and that of ^{47}Ca secreted into bowel lumen with the intestinal juices. This latter component of the calcium available for absorption is especially significant in the first days when specific activity of plasma calcium is decreasing rapidly (12). In view of the fact that some of the absorbed ^{47}Ca is secreted and lost in the feces, the extrapolate method offers theoretic advantage over the classic balance technique. In young rats or rats fed calcium-restricted diets in which absorptive efficiency is high and body retention increased, error introduced by secretion of ^{47}Ca during the initial several days is relatively small. In old rats and rats fed a surfeit of calcium, reabsorption of secreted calcium is minimal and potential magnitude of error is enhanced.

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Natural Occurrence and Biological Activity of Vitamin A Derivatives in Rat Bile [1]

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ABSTRACT A study was made to determine the biological activity of retinoyl β-glucuronide, a major biliary excretion product of administered retinoic acid in the rat, to evaluate the significance of the biliary excretion of vitamin A derivatives under normal nutritional conditions, to analyze the rate of excretion of vitamin A derivative, and to determine the chemical nature of the excreted products. Retinoyl β-glucuronide has a biopotency 30 to 100% as great as all-*trans* retinol or as all-*trans* retinoic acid. A more accurate estimate cannot be given because of the unknown effect of the isolation procedure on the glucuronide's biopotency. Similarly, a nonpolar retinoate derivative in bile (fraction I), which presumably is methyl retinoate, has a biopotency 50 to 200% as great as all-*trans* retinoate by like criteria. The biliary secretion of vitamin A derivatives, presumably glucuronides, appears to be a normal physiological process, inasmuch as labeled compounds were secreted at the rate of about 0.3 μg/ml in the bile of vitamin A-depleted rats from 6 to 32 days after the administration of labeled retinol. Finally, the rate of excretion of labeled retinoic acid derivatives in the excreta closely paralleled the rate of their secretion into bile. Most of the radioactivity appeared in the feces, mainly as retinoic acid but with a lesser amount as the glucuronide. A labeled derivative, presumably the glucuronide, appeared earlier in the urine but in much smaller amounts.

Retinoic acid,[3] although incapable of fulfilling the visual and reproductive functions of retinol and retinal, effectively stimulates the growth of vitamin A-deficient animals (2–4).[4,5] Although retinoic acid was not initially detected as a product of administered labeled retinol or retinal (5, 6),[6] it was later shown to appear transiently in several tissues shortly after the administration of retinal or retinol (7–9). Administered retinoic acid disappears quickly from the animal (3, 10),[7] however, and apparently is metabolized rapidly. Several reported biologically active metabolites of retinoic acid (5, 11, 12), although presumed to be degradation products of retinoate, have not been characterized chemically.

Alternatively, the rapid disappearance of retinoic acid from tissues may be attributed to its conjugation in the liver and intestine and the excretion of these conjugates in the bile (7, 8, 13–15). The major water-soluble conjugate of retinoic acid in bile has been identified as retinoyl β-glucuronide (16, 17).[8] Similar water-soluble compounds are excreted in the bile when retinol or retinal is administered (7, 8, 15, 18).

In the present investigation, the biological activities of retinoyl β-glucuronide and of a nonpolar ester of retinoate (fraction I) were assessed, the secretion of metabolites of vitamin A in the bile under normal nutritional conditions was examined, and the rate of excretion and nature of the excretory products after retinoic acid administration were determined.

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[3] The recommended nomenclature for vitamin A derivatives (1) and common synonyms are as follows: retinol (vitamin A alcohol), retinal (retinene, vitamin A aldehyde), retinoic acid (vitamin A acid), methyl retinoate (vitamin A acid methyl ester), retinoyl β-glucuronide (vitamin A acid β-glucuronide).

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EXPERIMENTAL PROCEDURE

Radioactive substrates. All-*trans* 6,7-^{14}C-retinol, all-*trans* 6,7-^{14}C-retinoic acid and all-*trans* 15-^{14}C-retinoic acid were used.[9] The compounds were diluted with appropriate amounts of crystalline all-*trans* retinol or all-*trans* retinoic acid,[10] and were purified by chromatography on deactivated alumina (8% H_2O) or silicic acid, respectively.

Metabolites of retinoic acid were purified by the following modification of reported procedures (15–17).

Preparation of bile samples. Five male normal rats (450-g weight, Rolfsmeyer strain) fitted with bile cannulae were injected with 6 to 8 mg of all-*trans* 6,7-^{14}C-retinoic acid. The total pooled bile was diluted with 10 volumes of methanol, and the mixture was shaken and centrifuged. The final precipitate, after being rewashed with methanol, had no radioactivity and was discarded. The pooled supernatant solution was evaporated to dryness under a partial vacuum, and the residue was extracted several times with small volumes of methanol. The radioactivity was soluble in methanol, whereas most of the inorganic salts were not.

Preparation of fraction I. The final methanolic extract of bile samples collected for 16 hours was absorbed on a small amount of silicic acid and the solvent was evaporated off. The absorbed extract was then transferred to the top of a 17.5-cm × 5-cm silicic acid column which had been equilibrated with petroleum ether. Gradient elution was performed with petroleum ether, diethyl ether, and ethanol. Fraction I was eluted by 10 to 25% diethyl ether in petroleum ether. Although the absorption spectrum was of pure retinoic acid, no free retinoic acid was detected. The R_F was similar to that of methyl retinoate in 2 solvent systems when analyzed by thin-layer chromatography (16, 17).

Preparation of retinoyl β-glucuronide (fraction III). The final methanol extract of bile samples collected for 24 hours was placed on a 15-cm × 4.5-cm column of Bio-Rad AG2-X8 anion exchange resin in the acetate form, and was eluted first with methanol and then with increasing amounts of glacial acetic acid in methanol. The methanol extract, which contained fraction I, and the first few fractions with acetic acid, which contained free retinoic acid, were rejected. The glucuronide fraction was evaporated to dryness, suspended in *n*-hexane, and chromatographed on a silicic acid column with a linear gradient of absolute ethanol in hexane. The polar compound this obtained showed a retinoic acid spectrum and behaved like retinoyl β-glucuronide by thin layer chromatography (16, 17). No detectable free retinoic acid was present.

Chromatography of all-trans retinoic acid. All-*trans* retinoic acid was chromatographed on silicic acid by the method used with fraction I. Retinoic acid was eluted by 5% ethanol in diethyl ether.

Preparation of deficient animals. Male weanling rats (40 to 50 g, 3 to 4 weeks old) of either the Holtzman or Rolfsmeyer strain were fed a USP vitamin A test diet.[11] Deficiency symptoms appeared within 5 to 7 weeks. The deficiency was characterized in both strains by respiratory trouble, keratinization of the eyes, porphyrin on the nose and paws, and finally a sudden loss of weight leading to death.

Bioassay. Assays are often made before late deficiency develops, for instance, when the rat's weight increases by less than 1 g per day for 4 successive days (4). In the present experiments an acute deficiency was assured by using only animals that lost weight significantly for 2 consecutive days. About half of these deficient rats died regardless of treatment. Growth responses were analyzed by the methods of Sherman and Todhunter (19) and of Moore (20).

Vitamin A and its derivatives were administered intraperitoneally in a micellar solution of polyoxyethylene sorbitan monooleate (Tween 80) and isotonic saline. The concentration of each retinoic acid derivative was calculated on the basis of its absorption at 355 mμ, assuming the extinction coefficient of retinoic acid conjugates to be the same as that of all-*trans* retinoic

[9] Kindly supplied by Hoffmann-La Roche, Inc., Basle, Switzerland.

[10] Obtained from Distillation Products Industries, Rochester, New York.

[11] The percentage composition of the USP vitamin A test diet was: starch, 65; vitamin-test casein, 18; dried yeast, USP, 8; vegetable oil, 5; salt mixture USP no. 2, 4; and viosterol (vitamin D_2), 0.5 g/45.5 kg of diet (Vitamin A Test Diet obtained from Nutritional Biochemicals Corporation, Cleveland).

acid. The amount of each retinoic acid derivative in bioassay studies is expressed in terms of the equivalent weight of retinoic acid in the compound.

Bile collection from rats treated with labeled retinol. Rolfsmeyer all male weanling rats were depleted of vitamin A with the USP vitamin A test diet. When a rapid loss of weight (10 g or more/7 days) was observed, the rats were injected with a total of 355 to 1125 μg of all-*trans* 6,7-^{14}C-retinol (2,200 dpm/μg), 150 μg/ml) in a solution of 0.1% Tween 80-isotonic saline. The surviving rats increased in weight and were cannulated at 6, 15, and 30 days. During the subsequent 48-hour collection of bile, rats were given either glucose-saline or water. Aliquots of the bile were counted in a Packard Tri-Carb liquid scintillation counter in the dioxane-naphthalene scintillation fluid of Bray (21). At the end of 48 hours, each rat was killed and the total radioactivity in the chloroform methanol extract of its liver was measured. Quenching in the yellowish solutions was corrected by adding internal standard (^{14}C-toluene) to each assay vial and recounting. The counting efficiency was 52 to 58%.

Analysis of urine and feces. An adult male Rolfsmeyer rat (284 g) was injected intraperitoneally with 27 μg of 15-^{14}C-retinoic acid (2×10^6 dpm). The carboxyl labeled compound was used in this case in preference to 6,7-^{14}C-retinoate because of its higher specific activity. Feces and urine samples were collected at different intervals. Aliquots of the urine were counted directly.

Each fecal sample was ground in a mortar with excess $(NH_4)_2SO_4$, brought to pH 4 with 1 N HCl and extracted 5 times with several volumes of *n*-butanol. The combined butanol extracts were evaporated to dryness. The residue from the butanol extract was then crushed and extracted 5 times with methanol. Since in control experiments 80% of the labeled retinoic acid added to unlabeled feces was recovered in this final methanol extract, observed values for labeled feces were corrected accordingly. Internal standards were used to correct for quenching. The counting efficiency was 62 to 66%.

RESULTS

Biopotency of biliary metabolites of retinoic acid. Retinoyl β-glucuronide, when administered at a dose of 27 μg/day for a 10-day period, gave the same response as that induced by 9 μg all-*trans* retinoic acid under identical conditions (fig. 1), as estimated by Sherman's method (19). When daily doses of 10 μg or 15 μg retinoyl β-glucuronide were given to 3 surviving rats for 10 days, however, no growth response was observed.

Since the isomerization of metabolites of retinoate during extraction and chromatography would markedly affect their biological potencies, all-*trans* retinoic acid was treated by the identical isolation procedure. The biopotency of the chromatographed retinoic acid was only about one-third that

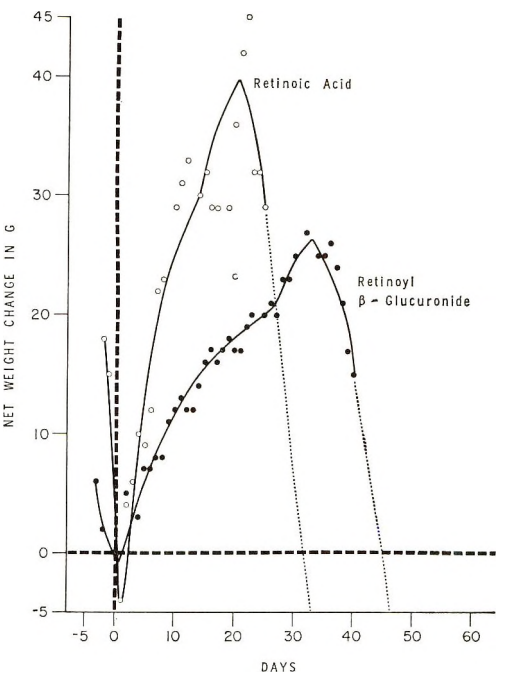

Fig. 1 Growth response of deficient rats of the Holtzman and Rolfsmeyer strain treated for a 10-day period with daily doses of 9 μg all-*trans* retinoic acid or of 27 μg of retinoic acid as retinoyl β-glucuronide. Three surviving rats were used in the glucuronide group, and 5 in the retinoic acid group. The area under the respective curves was either computed for total area or for individual net weights. When the areas under each curve were traced on graph paper, cut out and weighed, a value of 945 ± 6 mg was obtained for both curves.

of all-*trans* retinoate and of all-*trans* retinol (table 1). Similarly fraction I, given in a single dose of 20 μg or in repetitive doses of 10 μg for 5 days, was only about half as active as all-*trans* retinoic acid (table 1). When a single dose of 50 μg of fraction I was administered to 2 deficient rats, however, a relative potency of slightly more than 100% was observed in reference to all-*trans* retinoic acid.

Natural occurrence of vitamin A derivatives in bile. Since acutely deficient rats are essentially depleted of retinol, the specific activity of labeled derivatives in bile was assumed to be the same as that of the administered ^{14}C-retinol. On this basis the radioactivity in bile, after correction for quenching, is expressed as micrograms of labeled compound per ml. As shown in table 2, the concentration of retinol derivatives in the bile is relatively constant in all 4-hour periods from 6 to 32 days after retinol injection. However, the total bile flow and hence the total radioactivity in a given period varies considerably, and is related rather to the physical condition of

TABLE 1

Approximate biopotency of chromatographed retinoic acid and of fraction I

Treatment	Substance	Daily dose	No. of rats	Avg net wt gain	Area under time-weight curve [1]	Calculated biopotency
		μg		g		%
Single doses	all-*trans* retinoic acid	20	6	+31±4.4 [2]	363	100
	all-*trans* retinol	8	4	+20±6.5	152 [3]	105
	chromatographed retinoic acid	20	3	+18±6.3	120	33
	fraction I [4]	20	3	+21±1.5	196	54
5 daily doses	all-*trans* retinol	10	3	+77±7.8	657	100
	chromatographed retinoic acid	10	3	+24±1.6	181	28
	fraction I [4]	10	3	+50±9.1	376	57
Untreated	—	—	5	−33±3.3	0	—

[1] Plots were made of the average weight against time and areas are expressed in arbitrary units on the basis of weight.
[2] Averages ± SE.
[3] For a dose of 20 μg, the corresponding value would be 380.
[4] The weight of fraction I is expressed as the equivalent amount of retinoic acid in the compound.

TABLE 2

Concentration of radioactivity in bile of rats at various periods after injection of ^{14}C-retinol (2200 dpm/μg)

Collection period after cannulation	Time between the last retinol injection and cannulation					
	6 days		15 days		30 days	
	Bile volume	Labeled compounds	Bile volume	Labeled compounds	Bile volume	Labeled compounds
hours	*ml*	*μg/ml*	*ml*	*μg/ml*	*ml*	*μg/ml*
0–4	1.47	0.34	1.10 [2]	0.56 [2]	2.25	0.41
4–8	1.60	0.29	1.85	0.54	2.20	0.32
8–12	1.47	0.24	1.80	0.27	1.70	0.34
12–20	3.10	0.15	2.55	0.25	3.30	0.31
20–24	1.35	0.17	1.10	0.25	1.60 [3]	0.39 [3]
24–28	1.25	0.18	1.55	0.26	2.00	0.25
28–32	0.95	0.22	1.90	0.27	2.35	0.28
32–36	0.70	0.24	2.00	0.27	2.70	0.25
36–44	1.10	0.28	4.23	0.24	6.45	0.21
44–48	0.45 [2]	0.44 [2]	1.63	0.29	3.30	0.22
48–52	—	—	1.30 [2]	0.27 [2]	—	—

[1] Rats were cannulated 6, 15, and 30 days after the injection of all-*trans* 6,7-^{14}C-retinol and bile was collected for 48 hours. Two rats were cannulated after 6 days, 4 rats after 15 days, and 2 rats after 30 days. The radioactivity in bile, after correction for quenching, is expressed as micrograms of labeled compound derived from the administered retinol. The individual values agreed within 30% in most cases.
[2] Only two rats.
[3] Only one rat.

TABLE 3

Influence of dosage schedule and other factors on biliary excretion of labeled compounds [1]

Exp. no.	Net loss of wt [2]	No. of rats	^{14}C-retinol administered [3]	Time between last injection and cannulation	Net wt gain	Avg amount of labeled compound	
						In bile during 48-hr period	In liver after 48-hr period
	g		*μg*	*days*	*g*	*μg/ml*	*μg*
1	45	3	355	6	46	0.26 ± 0.09 [4]	34 ± 0.6
2	32	3	450	15	43	0.28 ± 0.01	12 ± 1.2
3	14	3	450	15	36	0.33 ± 0.02	30 ± 5.0
4	20	2	1125	30	83	0.30 ± 0.02	15 ± 1.4

[1] The specific activity of the injected 6,7-^{14}C-all-*trans* retinol was 2200 dpm/μg. Samples were corrected for quenching.
[2] During 9 days before vitamin A treatment.
[3] In experiment 1, the deficient rats were given the cited amount in seven doses during a 9-day period. In experiments 2 and 3, a single dose was administered. In experiment 4, a single dose of 225 μg was given initially; the surviving rats were then given an additional dose of 900 μg after 7 days.
[4] Means ± SE.

the cannulated rat and to his fluid intake. Under varying conditions of injection schedule, dose, residual vitamin A in the liver, and period between the last injection of labeled retinol and cannulation, the concentration of labeled compounds in the bile was remarkably constant, with an average excretion rate of about 0.3 μg/ml (table 3).

Bile samples were also analyzed by thin-layer chromatography, and the distribution of radioactivity was compared with the migration of several vitamin A compounds. As shown in table 4, over 80% of the radioactivity remained near the origin, with an R_F similar to that of known retinoyl β-glucuronide, whereas 10% migrated with the retinoic acid spot. Only trace amounts of radioactivity appeared in more polar compounds. In keeping with previous studies (13, 15) little retinol, if any, was excreted in the bile. Treatment of the whole bile with β-glucuronidase caused about a 10% decrease in the radioactivity of the major polar compound and a corresponding increase in the radioactivity of the retinoic acid fraction. Since yields in this assay are often poor in the presence of large amounts of lipid impurities, we can conclude only that at least a significant portion of the excreted radioactivity is a β-glucuronide of retinoic acid.

TABLE 4

Analysis of bile samples by thin-layer chromatography [1]

Distance from origin	Migration of standards [2]	Distribution of recovered radioactivity [3]
cm		%
2	Retinoyl β-glucuronide	83
4	Retinoic acid	10
6	Retinol	3
8		3
10		1

[1] Radioactive bile samples were evaporated to dryness (60°) and dissolved in 2 ml of 0.1 M acetate buffer. After the addition of excess methanol and unlabeled retinoic acid, the samples were dried, redissolved in methanol, and spotted in thin layer chromatography plates. The plates were developed in benzene:chloroform:methanol (4:1:1).
[2] Reference samples of retinoyl β-glucuronide and retinol migrated as indicated. Reference unlabeled retinoic acid was sometimes added to the sample and sometimes spotted separately.
[3] About 75% of the added radioactivity (543 dpm) was recovered from the TLC plate.

Analysis of urine and feces. Within 72 hours, 73% of the radioactivity administered as 15-^{14}C-retinoic acid was found in the feces and urine. Nearly 10% of the total counts were recovered from the urine and about 65% from the feces. The cumulative excretion rate in the urine and feces are compared in figure 2 with that of labeled compounds in the bile of bile duct cannulated rats. Three other experiments gave similar results, but with a somewhat lower recovery of radioactivity in the excreta. The rates of biliary excretion were essentially identical when either 15-^{14}C-retinoic acid or 6, 7-^{14}C-retinoic acid was injected, in full accord with past studies (15, 17).

Labeled compounds appeared immediately in the urine after the intraperitoneal injection of ^{14}C-retinoic acid, at least 6 hours before their appearance in the feces. As in the case of bile, extracts of the urine and feces were analyzed by thin-layer

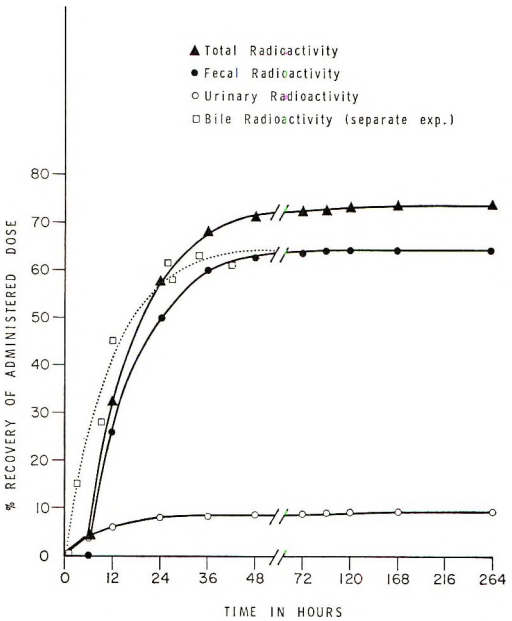

Fig. 2 The cumulative excretion rate of labeled compounds in the urine and feces after the injection of 15-^{14}C-retinoic acid. For comparison, the cumulative excretion rate in the bile of bile duct cannulated rats injected with 15-^{14}C-retinoic acid in a separate experiment is also given.

chromatography, and the distribution of radioactive compounds was compared with the migration rate of known vitamin A derivatives. In the case of urine, nearly all of the radioactivity migrated in the glucuronide zone, a small amount appeared in the retinoic acid area, and essentially no radioactivity was in less-polar compounds (table 5).

Of the labeled fecal products however, only about one-third of the radioactivity was present in the glucuronide zone, whereas 59% migrated with retinoic acid (table 5). A small amount, about 8% appeared in non-polar compounds. After methylation of the fecal extract with diazomethane, the largest fraction of the radioactivity migrated with methyl retinoate, whereas a significant amount (16%) appeared in the esterified glucuronide zone. An appreciable amount (22%) of radioactivity also was found between these 2 fractions (table 5). Whether this fraction represents additional unidentified acidic compounds in the original fecal extract, or is a mixture of the methyl esters of retinoic acid and its β-glucuronide, is not known.

DISCUSSION

Retinoyl β-glucuronide, administered intraperitoneally, stimulated growth markedly at high doses but was biologically inert at low doses. At present we can only speculate about the events underlying this phenomenon. Possibly the glucuronide was excreted rapidly compared with the time required for a growth response to be initiated, or for its hydrolysis to retinoic acid, which is a known growth promotor. Possibly the test animals were so acutely deficient that a threshold for vitamin A was established, or that the repairing processes leading to survival at low doses com-

TABLE 5

Analysis of labeled products of retinoic acid in urine and feces by thin-layer chromatography

Distance from origin	Migration of standards [1]		Distribution of recovered radioactivity [2]		
	Free acids	Methyl esters	Urine untreated	Feces untreated	Feces methylated
cm			%	%	%
2	Retinoyl β-glucuronide		95	32	8
4	Retinoic acid		4	59	15
6		Retinoyl β-glucuronide Methyl ester	1	5	16
8			0	3	22
10		Methyl retinoate	0	1	39

[1] Samples were chromatographed on plates of silica gel C developed with benzene:chloroform:methanol (4:1:1). The migration rates of reference retinoyl β-glucuronide and its methyl ester are based on reported values; retinoic acid and its ester were run as controls either with the sample or separately.

[2] The radioactivity spotted on the TLC plate was: urine (4942 dpm), untreated feces (6617 dpm) and methylated feces (7164 dpm). The amount recovered was 78%, 63% and 50%, respectively.

peted unfavorably with degradative ones leading to extinction. Whatever the explanation, this observation may clarify the heretofore puzzling fact that all polar metabolites of retinoate isolated by others (5, 11) from rat liver and intestine were inert biologically. Thus these yet uncharacterized water-soluble compounds, rather than being degradation products of retinoic acid, might well be glucuronides of vitamin A which were not administered in an adequate dose to give a growth response. The further characterization of these compounds will be awaited with interest.

The biological activity of retinoyl β-glucuronide was between 30 and 100% of that of all-*trans* retinol and *all*-trans retionic acid. This broad estimate of biopotency was mainly due to the uncertain effect of the isolation procedures on the biopotency of the compound. When all-*trans* retinoate was treated by similar procedures, its bioactivity was reduced to 30%, probably as a result of isomerization during chromatography. Several *cis*-isomers of retinoic acid, for example, are known to have a markedly lower biopotency than the all-*trans* form (21, 23). Precise evaluation of the bioactivity of all-*trans* retinoyl β-glucuronide must probably await its chemical synthesis.

The biologically active nonpolar derivative of retinoic acid isolated from fraction I is most likely methyl retinoate (24),[12] a known stimulator of growth (3, 25). Although methyl esters of some fatty acids might well occur in nature (26, 27), methyl retinoate of bile is probably an artifact arising from the transesterification of retinoyl β-glucuronide with methanol during isolation.[13] In any event its biopotency in these experiments was 50 to 200% that of all-*trans* retinoic acid, depending again on the effect of the isolation procedure on its biopotency. Under some conditions methyl retinoate is known to have a higher biopotency than retinoic acid itself (3).

In most past studies dealing with the biliary excretion of retinoyl β-glucuronide, relatively large doses of vitamin A were injected intraportally and the bile was immediately collected (15, 17). Whether the biliary excretion of the glucuronide was only a detoxication mechanism for large doses of vitamin A, or was rather a normal physiological process, was uncertain. In the present experiments, rats were shown to excrete labeled compounds in the bile at the rate of about 0.3 μg/ml bile from 6 to 32 days after the injection of 355 μg to 1125 μg labeled retinol. Although the bile flow in a bile-duct cannulated rat may differ appreciably from that in a normal animal, the approximate excretion rate of retinol derivatives in this study is about 3 μg/day. In analogous studies with rats dosed with about 50 μg of 15-^{14}C-retinol, the excretion rate in the bile after 36 to 40 hours was linear and equal to approximately 1 μg/day (7). The biliary excretion of vitamin A derivatives appears, therefore, to be a well-controlled physiological process. In view of the small amount of radioactivity present in bile in the present study, however, characterization of the biliary compounds was difficult. Nonetheless, the major portion of the radioactivity was found in polar compounds similar to vitamin A glucuronide, and not in either free retinol or free retinoic acid.

Metabolites of intraperitoneally injected ^{14}C-retinoic acid were excreted in the feces and urine at a rate similar to that of their secretion in bile. Thus these biliary metabolites, although involved in an enterohepatic circulation (15), apparently are not well conserved within the animal. Labeled metabolites first appeared in the urine, presumably as the glucuronide, and later in the feces, largely as free retinoate but also as a polar compound which is probably the glucuronide. That water-soluble metabolites of retinol are present in urine and that the feces are the major excretory route for retinol metabolites has long been known (28). In a recent study about 27% of the administered dose of 15-^{14}C-retinoic acid was found in the urine within 24 hours, at least in part as retinoyl β-glucuronide (7). Our results accord with these observations, except that the percentage excreted in the urine in our study was somewhat lower (ca. 10%). The percentage of the recovered dose excreted in the feces varies from 40 to 65%, depending in part on whether retinol or retinoic acid is injected and on the label-

[12] Lippel, K. 1966 Biosynthesis and characterization of metabolites of retinoic acid in the rat. Ph.D. thesis, University of Florida.

[13] See footnote 12.

ing pattern of the substrate (29). In our study a maximum of about 65% of the administered dose of 15-^{14}C-retinoic acid was found in the feces, in reasonable agreement with this report (29).

Since relatively large amounts of retinoic acid are present in feces after the intraperitoneal administration of retinoate, coprophagy may have an important influence on nutrition experiments involving vitamin A under certain conditions. In fact, the growth of vitamin A-deficient rats was found to be stimulated by their ingesting dried feces from a normal animal injected with retinoic acid.[14] The free retinoic acid present in feces presumably arises from hydrolysis of the glucuronide by β-glucuronidases present in the enterobacteria.

Thus, the physiological events involving vitamin A in the rat might be summarized as follows: Ingested retinol is absorbed, esterified, and transported via the lymph to the liver, where it is stored mainly as retinyl ester. After hydrolysis of the ester, retinol is partially transported to other tissues, partially conjugated to the β-glucosiduronate,[15] and partially oxidized to retinoic acid. The latter may in part be decarboxylated and further degraded (29), or may form a β-glucuronide, which is secreted in the bile. A portion of the glucuronides are returned to the liver by an enterohepatic circulation, but the major portion ultimately is excreted in the feces, with some in the urine. During its passage through the lower bowel, retinoyl β-glucuronide is largely cleaved to the free acid, presumably by bacterial action.

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[15] See footnote 12.

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Antidystrophic Effect of Selenium and Other Agents on Chicks from Vitamin E-depleted Hens [1]

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ABSTRACT A study was undertaken to determine the relation of dietary lard and body vitamin E stores to the protective efficacy of selenium against nutritional muscular dystrophy in the chick. Flocks of vitamin E-depleted hens were used for the production of eggs with very low levels of α-tocopherol. Chicks hatched from these eggs and fed a purified diet containing negligible traces of vitamin E had indeterminably low stores of α-tocopherol after the 5-week experimental period. Selenium supplementation of the "fat-free" basal diet provided partial protection against myopathy in the severely vitamin E-depleted chicks indicating that at least part of the antidystrophic activity of the element does not require vitamin E. Selenium protectivity was markedly inhibited in both vitamin E-deficient and control birds by the inclusion of 4% of lard in the diet. These findings confirm previous observations from this laboratory that 4% of lard, or its equilavent content of linoleic acid, may act as a selenium antagonist. The present data suggest that in the chick, lard (or linoleic acid) may directly interfere with the antidystrophic activity of selenium rather than solely by the destruction of feed and tissue vitamin E.

It now appears well established that most of the enzootic muscular dystrophies in lambs (1–5) and calves (5–7) can be prevented by selenium administration. However, it is also evident that the myopathies induced experimentally in these animals by feeding diets containing unsaturated fats are relatively unresponsive to the element (8, 9). Similarly, nutritional muscular dystrophy in laboratory animals, which has been almost exclusively produced by the use of vitamin E-deficient diets containing unsaturated fat, is not prevented by supplements of selenium (10–13). These observations suggest that the effectiveness of selenium in preventing muscle degeneration is in some way inhibited by the presence of unsaturated fat in the diet.

Experiments conducted in this laboratory on the etiology of myopathy in the chick showed that in this species unsaturated lipid is not required for production of the disorder. It was observed that when "fat-free" diets were used, selenium provided complete protection (14). However, when 4% of lard was fed, or the linoleic acid equivalent of 4% of lard, even toxic levels of the element (5 ppm to 10 ppm) provided only partial protection (15). Subsequent studies have revealed that the eicosapentaenoic and docosahexaenoic fatty acids of cod liver oil also inhibit selenium activity.[4]

As synergistic relationships exist between selenium and vitamin E in the prevention of muscular dystrophy in the chick (14, 16), the above observations pose the question as to whether the observed inhibition of selenium activity by unsaturated fat is (a) simply the result of vitamin E destruction in the gastrointestinal tract or the tissues of the experimental animals, or (b) involves a direct interference in the metabolism of the element. In the study reported herein, chicks severely vitamin E-deficient were used in an attempt to answer this question.

EXPERIMENTAL

The chicks used in the present study were obtained from 4 flocks of White Plymouth Rock breeding hens maintained in the Department's facilities. Three flocks were fed a vitamin E-deficient basal diet

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[4] Unpublished observations, K. J. Jenkins and L. M. Ewen, 1964.

TABLE 1

Composition of vitamin E-deficient basal diet for hens

	g/100 g diet
Dextrose	57.13
Soybean oil meal (50% protein)	33.40
Ground limestone (38% Ca)	3.50
$CaHPO_4 \cdot 2H_2O$	3.00
Brewer's yeast	2.00
Salt (iodized, 0.015% I)	0.50
Vitamin premix [1]	0.44
$MnSO_4 \cdot H_2O$	0.014
$ZnSO_4 \cdot H_2O$	0.012

[1] Supplied the following: (mg/100 g diet) niacin, 0.44; riboflavin, 0.22; pantothenic acid, 0.22; D-biotin, 0.007; menadione, 0.05; pyridoxine·HCl, 0.20; vitamin B_{12}, 0.0004; vitamin A palmitate, 441 IU; vitamin D_3, 45 ICU; and choline chloride, 44.

similar to that of Singsen et al. (17), 2 flocks of which were given supplements of either 0.3% of corn oil or 50 ppm of α-tocopheryl acetate (table 1). The fourth group of hens received a commercial breeder's ration. The birds were maintained in wire-floor pens in groups of ten. Roosters penned with the hens were alternated weekly between the various low and adequate vitamin E groups to avoid depletion of their tissue tocopherol stores. Feed and water were supplied ad libitum. After a 6-week preliminary feeding period the eggs were collected, stored at 10° in a constant temperature chamber and incubated at weekly intervals.

One-day-old male and female chicks were distributed at random among the various dietary treatments and maintained in wire-floor, electrically heated battery brooders with feed and water provided ad libitum. The cystine- and vitamin E-deficient chick diet used in all dietary treatments, which contained 20% of purified casein,[5] 1% of L-arginine·HCl, and no added fat, has been described previously (18). At the end of the 5-week experimental period the birds were killed and examined visually for incidence and severity of muscular dystrophy in the pectoralis muscles.

Tocopherol determinations were carried out on eggs collected at the starting, median, and final stages of the collection period as well as on carcasses of 5-week old chicks at the termination of the experiment. The vitamin E method used was that of Green et al. (19) with minor modifications as suggested by the Vitamin E Panel, Analytical Methods Committee (20).

RESULTS AND DISCUSSION

No difficulty was encountered in the production of chicks from severely vitamin E-depleted hens. Eggs from hens receiving the basal ration averaged 73% hatchability based on the number of eggs set. Similarly, the hatchability of eggs from hens that received the basal ration plus vitamin E or the commercial breeder's ration averaged 85%, and 67%, respectively, for the 14-week depletion period. The average α-tocopherol content of the eggs from vitamin E-depleted hens was very low, ranging from 0.6 ppm to 1.8 ppm; the control hens produced eggs containing 23 ppm to 34 ppm α-tocopherol (table 2). No tocopherol (less than 0.5 ppm) was detectable in the carcasses of 5-week old chicks derived from the vitamin E-deprived hens.

The results (table 2) confirm previous observations (14) that supplementation of selenium to a "fat-free" diet markedly reduces the incidence of muscular dystrophy in chicks derived from hens provided with vitamin E. Chicks severely vitamin E-deficient also were protected by selenium administration, although to a lower degree. These latter data demonstrate that selenium has a role in the prevention of muscular dystrophy in the presence of neglibible intakes and tissue stores of vitamin E. Other studies in this laboratory [6] also have shown the converse, that is, tocopherol will prevent the disorder in the presence of very low dietary levels of selenium (that is, with diets that will promote the occasional development of exudative diathesis). It is not possible from the data to conclude whether selenium and tocopherol have different functions in the prevention of the chick dystrophy. The confirmed reports (14, 16) of synergism between these agents suggests that their modes of action are closely related.

When 4% of lard was fed to control chicks receiving 2.0 ppm selenium, a marked increase in the incidence of dystrophy resulted. This observation confirms similar findings reported previously by

[5] Vitamin-Free Casein, Nutritional Biochemicals Corporation, Cleveland.

[6] Unpublished observations, K. J. Jenkins, 1962.

TABLE 2

Influence of maternal diet on the efficacy of selenium and other protective agents against muscular dystrophy in the chick

Supplements to chick basal diet [1]	Maternal diet											
	Basal [2]			Basal + 50 ppm vitamin E [3]			Basal + 1% corn oil [4]			Commercial breeder's [5]		
	Muscular dystrophy											
	Incidence [6]		Severity [7]	Incidence		Severity	Incidence		Severity	Incidence		Severity
		%			%			%			%	
Nil	46/46	100	546	24/51	47	92	44/49	90	402	90/100	90	402
0.1 ppm Se [8]	—	—	—	—	—	—	32/38	84	341	38/79	48	111
0.5 ppm Se	23/42	55	168	4/48	8	5	16/34	47	115	13/78	17	34
2.0 ppm Se	15/42	36	124	5/51	10	9	7/25	28	116	3/30	10	23
4% lard [9]	37/38	97	493	35/40	88	421	—	—	—	—	—	—
4% lard + 2.0 ppm Se	41/44	93	411	38/53	72	218	19/22	86	302	22/30	73	277
0.08% L-cystine	11/30	37	142	2/24	8	29						
0.08% L-cystine + 0.5 ppm Se	5/29	17	50	1/24	4	4						
0.08% L-cystine + 4% lard	22/28	79	289	20/25	80	328						
0.08% L-cystine + 4% lard + 0.5 ppm Se	19/28	68	295	10/24	42	81						
0.1% DL-methionine	18/30	60	252	9/25	36	60						
0.1% DL-methionine + 0.5 ppm Se	4/28	14	43	3/24	12	8						
0.1% DL-methionine + 4% lard	24/27	89	344	19/24	79	294						
0.1% DL-methionine + 4% lard + 0.5 ppm Se	13/28	46	109	14/24	58	156						
0.1% DPPD [10]	11/14	79	236	5/17	29	32						
0.1% DPPD + 0.5 ppm Se	2/13	15	8	1/15	7	3						
0.1% DPPD + 4% lard	11/13	85	315	14/16	88	344						
0.1% DPPD + 4% lard + 0.5 ppm Se	8/12	67	233	6/14	43	43						
	Avg [11] α tocopherol content of eggs, ppm											
Hen depletion time, 6 weeks	1.4			33.5			1.8(2.2)			24.1(26.5)		
10 weeks	0.8			28.7			0.6			—		
14 weeks	0.7			28.9			0.6			22.7		

[1] Supplements replaced an equivalent amount of dextrose in the basal diet.
[2] Composition of basal diet for hens given in table 1.
[3] Added as Myvamix, a concentrate containing 20,000 IU of *d*-α-tocopheryl acetate/454 g; obtained from Distillation Products Industries, Rochester, New York.
[4] Mazola, Best Foods Division of the Canada Starch Company, Ltd., Montreal, Canada.
[5] Obtained from Feed Division, United Co-operatives of Ontario, Guelph, Ontario; contained 5% of fat, 0.25% of Santoquin, and 10 ppm of added vitamin E.
[6] Incidence = fraction of surviving chicks showing visible breast muscle lesions.
[7] Severity = $\frac{\text{total group score } (\times 100)}{\text{no. of surviving birds}}$. The birds were scored according to the incidence and severity of visible white striations in the breast muscles using a scale of 1–7.
[8] As Na_2SeO_3.
[9] Low vitamin E, molecular-distilled lard obtained from Distillation Products Industries.
[10] Diphenyl-*p*-phenylenediamine.
[11] For each value shown, 8 to 10 eggs were combined for a composite sample. Vitamin E determinations were run in triplicate and averaged. Value in parenthesis represents total tocopherol content where non-α-tocopherols were present.

Calvert et al. (16, 21) and Hutcheson et al. (14) that selenium is relatively ineffective against myopathy when fed with lard or linoleic acid. Of particular interest in the present study is the observed inhibition of selenium activity by lard when the element was administered to the vitamin E-deficient chicks. These data suggest that at least part of the effect of lard (linoleic acid) involves an antagonism of selenium activity not related to destruction of vitamin E. Previous studies (14) have shown that lard will reduce the protective efficacy of vitamin E as well as selenium. However, the important difference between the effects is that elevated dietary levels of tocopherol will prevent the disorder (18) while large increases in the selenium intake are ineffective (15).

In addition to selenium, low levels of the sulfur amino acids also were effective in preventing dystrophy in the vitamin E-depleted chicks. The presence of tocopherol stores further enhanced cystine and methionine activity. Supplementation with selenium in addition to cystine or methionine provided additional protection in both the control and "vitamin E-free" chicks confirming the previous observation that selenium provides protection per se (that is, does not simply enhance tocopherol activity).

Supplementation of the basal diet with 0.1% of DPPD had no appreciable influence on the development of lesions in "vitamin E-free" chicks but provided considerable protection in control birds. These data suggest that the synthetic antioxidant mediated its protective activity through vitamin E. When selenium and DPPD were provided together, DPPD enhanced the protective efficacy of selenium in the absence of vitamin E stores indicating that the synergistic relationship of vitamin E to selenium may be related to an antioxidant property of the vitamin.

In summary, the data presented in this report suggest that selenium may have a per se role in the prevention of muscular dystrophy in the chick; previous results from this laboratory (14) and others (16, 22), nevertheless, indicate that the protective mechanisms of selenium, α-tocopherol, and the sulfur amino acids are closely interrelated. Dietary linoleic acid increases the levels of α-tocopherol, synthetic antioxidants and the sulfur amino acids required to prevent muscular dystrophy. However, linoleic acid inhibition of selenium activity is more pronounced; the effect is not reversed by administering increased levels of the element (15, 21).

In view of the numerous reports that muscular dystrophy induced experimentally in lambs (9), calves (8), and laboratory animals (10–13) by the use of diets containing unsaturated fat, cannot be prevented by selenium administration, it should be of interest in future studies to establish whether a linoleic acid-selenium antagonism exists in the non-avian species as well.

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Calcium Status Studies on Adult Sheep [1]

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ABSTRACT Experiments were conducted with sheep, maintained with a calcium-deficient diet, to evaluate methods for determining calcium status of mature animals. During a 10-week balance trial, animals fed the Ca-deficient diet exhibited a linear decrease in plasma calcium for 3 weeks, after which it returned to the initial level and remained so for the next 7 weeks. Plasma phosphorus increased with time, but urinary phosphorus excretion did not increase. After the balance trial, the Ca-deficient sheep were given, via intravenous injection, 100 mg and later 300 mg of phosphorus. In comparison with controls the treated sheep were observed to maintain lower plasma phosphorus peaks immediately after the injection of phosphorus. Renal arterial-venous difference in phosphorus levels were greater in Ca-deficient animals, and hence it is postulated that renal phosphorus clearance was greater in the Ca-deficient sheep.

Assessment of calcium status in adult animals is difficult because these animals are able to draw upon skeletal reserves to meet body requirements (1). The adult animal, fed normal rations, exhibits higher endogenous losses, poorer absorption and poorer utilization of dietary calcium than young animals, which need dietary calcium for bone formation (2, 3). As there could be times when Ca-deficient diets are fed for extended periods, it would be desirable to have simple response criteria for use in assessing the calcium status in adult animals. The present studies were designed to evaluate response criteria, which might have value in the area.

EXPERIMENTAL PROCEDURE

Eight wether sheep weighing about 36 kg and which were more than one year of age were placed in metabolism stalls and fed a basal diet, the composition of which is shown in table 1, containing 0.01% calcium. Feces and urine were collected quantitatively daily for 70 days and the daily aliquots were combined into 10 consecutive 7-day periods. Blood samples, obtained by jugular vein puncture were taken initially and at the end of each 7-day balance period.

When the metabolism phase was completed, the eight treatment animals were paired with control sheep, which had been fed a commercial pelleted diet and housed in the metabolism room. All sheep received, via the jugular vein, 20 ml of a solution containing 100 mg of phosphorus, supplied as KH_2PO_4. The phosphorus solution was injected into the vein on one side of the neck and samples were obtained for analysis from the opposite vein at definite intervals of time after phosphorus injection. One week later, 2 sheep from both the Ca-deficient and control groups were injected with 300 mg of phosphorus following the above details. Two weeks later, 4 animals from each of the treated and control groups were anesthesized with halothane and the renal artery and vein cannulated for collection of blood samples. Each animal then received 500 ml of physiological saline via a femoral cannula and

TABLE 1
Composition of diets

	%
Ground corn	87.00
Urea	1.50
Cottonseed hulls	9.29
Calcium carbonate [1]	1.00
Sodium chloride	0.50
Potassium carbonate	0.71
	g/45.4 kg
Trace mineral mix [2]	7
Vitamins A and D [3]	10

[1] Omitted from calcium-deficient diet and replaced with hulls.
[2] Clifford et al. J. Animal Sci., 26: 400, 1967.
[3] The mixture supplied 20,000 IU and 2,500 USP units of vitamins A palmitate and D_2, respectively/g. Nopco, Harrison, N. J.

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initial blood samples were collected. Immediately afterwards 300 mg of phosphorus were injected into the femoral vein, and this was followed by a collection of blood samples from the renal vein and artery at one-minute intervals after the phosphorus injection.

Feed, feces, urine and blood were analyzed for calcium by atomic absorption spectrophotometry using the method of Willis (4). All phosphorus analyses were for inorganic phosphorus, using the method of Fiske and Subbarow (5).

RESULTS AND DISCUSSION

Table 2 shows the results of feeding a calcium-free diet for ten consecutive weeks on the balance of calcium and phosphorus during this period. Feed consumption was good throughout the experiment, thus the intake of calcium and phosphorus was fairly constant during all periods. Fecal calcium excretion was high during the first week, but was relatively constant for the next 9 weeks. As the sheep were receiving a complete diet containing 0.4% calcium just before being placed in the metabolism stalls, much of the fecal calcium during the first balance period must have come from the previous diet. As sheep do not excrete much calcium in the urine, calcium balance results followed the trends of fecal calcium.

Urinary phosphorus values did not differ ($P < 0.05$) during the entire trial. Fecal phosphorus values are characterized by two definite peaks during the first and fourth weeks; these peaks were higher ($P < 0.01$) than at all other periods. Partial explanation for the first peak has been offered but the authors have no adequate explanation for high excretion found during the fourth week. Results which could bear on the question are presented in figure 1, in which are presented results of feeding a Ca-deficient diet on plasma calcium and phosphorus levels: Plasma calcium decreased in a manner which did not differ from linearity during the first 3 weeks, but by the end of the fourth week the level had returned to that obtained initially and remained so for the duration of the experiment. These results are in accord with those obtained in an earlier experiment [3] in which rats were used. In another experiment,[4] it was found that the plasma calcium, after the initial decrease returned to normal and remained high in a sheep fed diet 1 for 23 weeks. The increase in plasma calcium level is in general agreement with the idea (6) that the lowest level of plasma calcium at the end of the third week stimulated parathyroid hormone secretion, which caused the increase in plasma calcium level observed at the end of the fourth week. If the classical mode of action of parathyroid hormone is to cause increased excretion of urinary phosphorus (7) it is notable that urinary phosphorus excretion did not increase during the fourth week and remain higher during the remainder of the experiment. It is of interest that instead, there was a great increase in fecal phosphorus excretion during the

[3] Nelson, T. E., J. G. Buchanan-Smith, A. J. Clifford and A. D. Tillman 1966 Mineral metabolism on a calcium-deficient diet. J. Animal Sci., 25: 893 (abstract).

[4] Unpublished data, Nelson and Tillman.

TABLE 2

Effect of feeding a calcium-deficient diet on excretion and retention of calcium and phosphorus

Periods	1	2	3	4	5	6	7	8	9	10
Days	0–7	7–14	14–21	21–28	28–35	35–42	42–49	49–56	56–63	63–70
					Calcium [1]					
Intake, g	0.39	0.38	0.39	0.40	0.42	0.42	0.43	0.40	0.36	0.35
Feces, g	8.8	3.3	3.0	2.8	3.4	5.2	3.6	4.0	3.6	2.5
Urine, mg	15.4	19.0	10.8	18.1	18.9	12.2	11.2	10.6	8.6	7.4
Retention, g [1]	−8.43	−2.93	−2.62	−2.42	−3.00	−4.99	−3.18	−3.61	−3.33	−2.16
					Phosphorus [1]					
Intake, g	9.0	8.7	8.9	9.2	9.7	9.6	9.7	9.6	7.2	
Feces, g	14.0	7.6	8.9	14.5	8.3	7.8	7.8	6.3	6.3	
Urine, g	3.7	2.2	2.8	2.6	2.9	2.3	3.3	2.6	1.9	
Retention, g [1]	−8.7	−1.1	−2.8	−7.9	−1.5	−0.5	−1.4	+0.7	−1.0	

[1] Average/week.

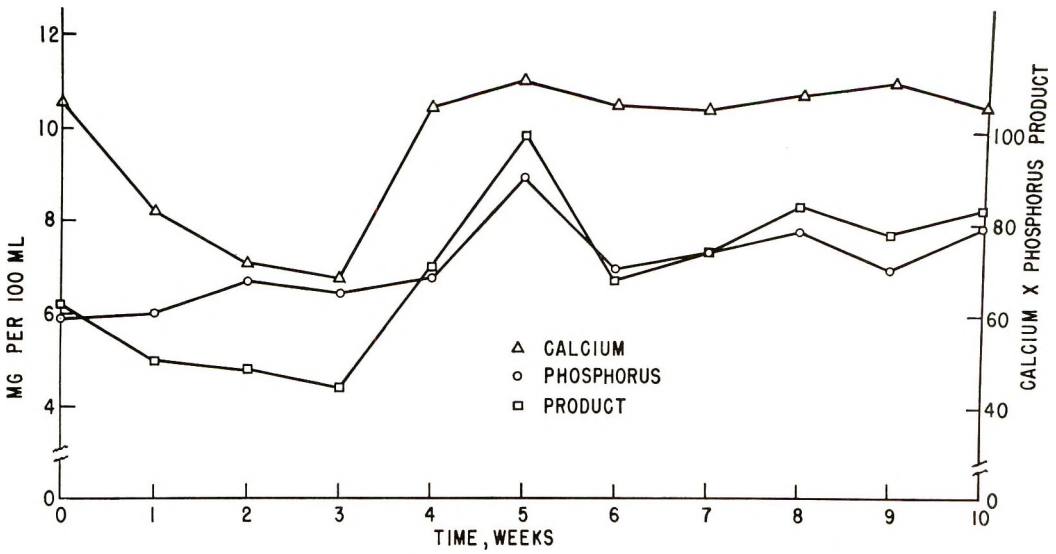

Fig. 1 Effect of a Ca-deficient diet on plasma calcium and phosphorus levels and Ca × P product in plasma.

fourth week. Whether the increase in plasma calcium level is related to the increased fecal phosphorus excretion noted during the same period is an open question which needs further investigation using more refined techniques. Plasma phosphorus level increased with time on diet, a phenomenon noted and explained by other workers (8). The product of total plasma calcium and plasma inorganic phosphorus describes a triphasic curve over the experiment and agrees with the results that Krook and Lowe (8) obtained with horses. The sequence of events in this curve was as follows: 1) During the first decreasing phase, hypocalcemia exerted a greater effect than did hyperphosphatemia. 2) During the first increasing phase, hyperphosphatemia exerted a greater effect than hypocalcemia. 3) In the second decrease, the body appeared to be compensating to a greater degree for hyperphosphatemia.

It is generally considered that a normal level for plasma or serum calcium is in the range of 9 to 12 mg/100 ml (9); thus an anlysis for plasma calcium between 7 and 21 days would have indicated a dietary deficiency. After 28 days the product of plasma calcium and plasma inorganic phosphorus increased beyond the initial level and the data indicate that a product higher than 70 could indicate an inadequate level of dietary calcium. In this respect, Krook and Lowe (8) obtained similar results when horses were fed an adequate level of calcium but with abnor-

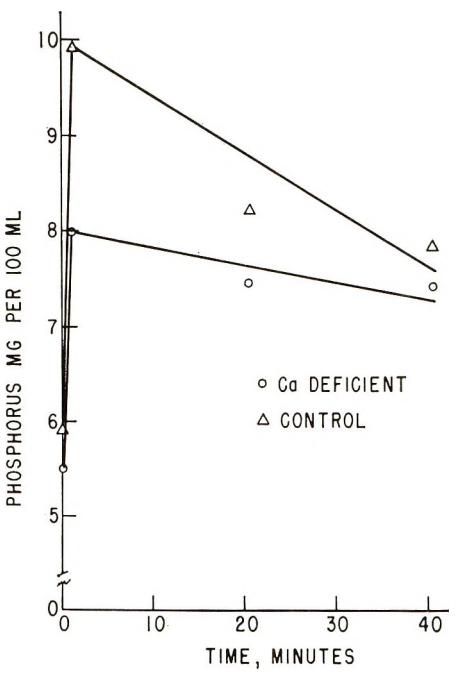

Fig. 2 Effect of an intravenous injection of 100 mg phosphorus upon subsequent plasma phosphorus levels of control and Ca-deficient sheep.

mal calcium-to-phosphorus ratio of 1:3 and 1:6.

When Ca-deficient sheep were injected intravenously with 100 mg of phosphorus, their rate of elimination of phosphorus was the same as that of the control group (fig. 2); however, the Ca-deficient animals maintained lower plasma inorganic phosphorus levels. These results indicated that a larger quantity of phosphorus so injected might show a greater difference between the Ca-deficient and control sheep; however, when 300 mg of phosphorus were injected the nature of the response (fig. 3) was the same as that with the lower level.

As the Ca-deficient sheep were able to keep the plasma inorganic phosphorus level lower than control sheep following an intravenous injection of either 100 or 300 mg of phosphorus, it was indicated, in keeping with the classic idea of parathormone function, that the treated sheep were able to excrete more phosphorus in the urine. When renal A-V differences were measured (fig. 4), it was found that the Ca-deficient sheep had a

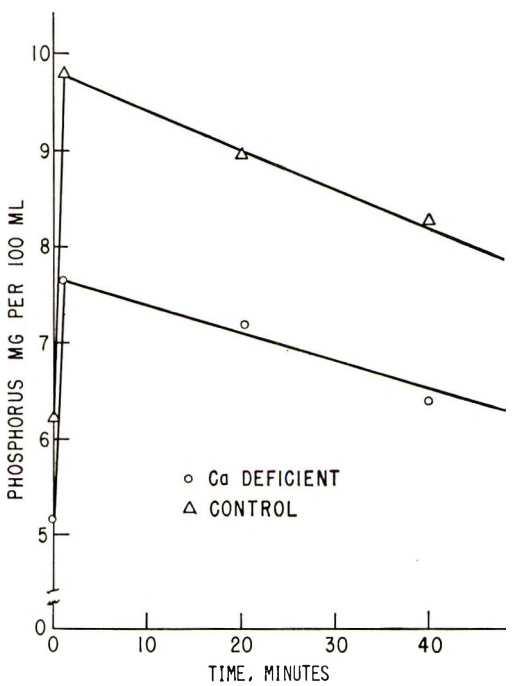

Fig. 3 Effect of an intravenous injection of 300 mg phosphorus upon subsequent plasma phosphorus levels of control and Ca-deficient sheep.

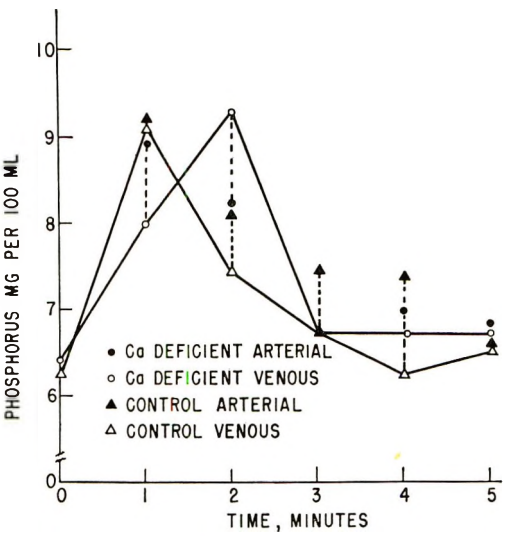

Fig. 4 Effect of an intravenous injection of 300 mg phosphorus upon A-V differences in plasma phosphorus.

significantly wider A-V spread ($P < 0.05$) at one minute after injection, but afterwards there were no significant differences between treatments. These results indicate that the mechanism for elimination of excess phosphorus from the blood stream in Ca-deficient sheep acts more rapidly than that of the control sheep. The response observed reflects an immediate one to a high, acute injection of phosphate. It does not necessarily follow the response of sheep to the Ca-deficient diet, where in this trial phosphorus was excreted mainly in the feces. The mechanism might be of such magnitude as to provide a method of determining the calcium status in sheep and suggests that a measure of parathormone activity might provide a more sensitive test. Such studies are presently underway in this laboratory.

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Thiamine Requirement of the Guinea Pig and the Effect of Salt Mixtures in the Diet on Thiamine Stability

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ABSTRACT The thiamine requirement of young guinea pigs and thiamine stability in semipurified diets for guinea pigs were investigated. The minimal level of thiamine·HCl supplementation which supported maximal gains, performance, and survival was in the range of 2.0 to 2.3 mg/kg of diet, depending upon the level of salt mixture. Acutely deficient animals showed poor growth, rough hair coat, anorexia, reduced food consumption, watery eyes, diarrhea, head retraction, unsteady gait, hyperextended hind legs, slobbering, and eventually, death. Symptoms were noted after feeding the deficient diet for about 2 weeks. Control animals fed thiamine·HCl had none of these symptoms. To minimize destruction of thiamine in the experimental diet, a new salt mixture, designed specifically for guinea pigs fed casein diets, was formulated and used successfully at 6% and 9% of the diet. At the 6% level thiamine stability in storage at 4° was optimal. Several other salt mixtures commonly used in guinea pig diets were compared for destructive effects on thiamine stability in refrigerated diets and all were found more destructive than the newly formulated mixture.

Very few studies have been made on thiamine deficiency in the guinea pig and little or no information, obtained by direct study, exists on the quantitative requirement of this vitamin for this species.[4] The earliest reports, made between 1939 and 1945, on a qualitative need for "vitamin B_1" by the guinea pig (1–3) cannot be considered conclusive, since control animals were not fed the pure vitamin and the studies were made with crude rations obviously deficient in many other nutrients as well, according to today's standards.

Reid (4), in 1954, was probably the first to obtain an uncomplicated deficiency of thiamine in the guinea pig and to describe symptoms of slow growth, emaciation, weakness, tremor, spasm, and death. Her control animals were given 16 mg of thiamine/kg of diet and appeared normal. Aptekar (5) has studied the effect of changes in the levels of dietary fat, carbohydrate, and protein on the severity of thiamine deficiency in guinea pigs but did not study mineral mixtures or quantitative requirements of the vitamin.

Earlier, in the first attempt to estimate the thiamine requirement of the guinea pig, Slanetz (6) had suggested that the requirement was not over 4 mg/kg of diet, since this was the amount he found (by rat assay) in an adequate commercial guinea pig ration. He made no direct studies on the thiamine requirement by use of the guinea pig. Reid (7) stated in 1962 that "the thiamine requirement for growth of young guinea pigs has been found to be between 6 and 8 mg per kg of diet" (referring to unpublished data). Most experimental diets, commonly used at present by investigators studying the guinea pig, contain 8 to 16 mg/kg of diet of thiamine to cover what has appeared to be an unusually high requirement for this vitamin. To determine whether this large amount is necessary was one of the purposes of this study.

Several investigators have reported salt mixtures as being a primary cause of thia-

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[4] After this study was completed, we learned of an independent study on the requirement of thiamine in guinea pigs by M. E. Reid and J. G. Bieri. (See Reid, M. E., and J. G. Bieri, Proc. Soc. Exp. Biol. Med., *126*: 11, 1967.) Their results on the level of thiamine required are substantially in agreement with those presented here.

mine instability in the storage of diets for rats (8–10). Lyman and Elvehjem (8) reported that KI, in the presence of $CuSO_4$ and ferric citrate, contributed to the instability of thiamine in diets in storage. Later, Waibel et al. (11) demonstrated that K_2HPO_4 was a primary ingredient in salt mixtures responsible for thiamine destruction and showed further that $CaCO_3$ and $MnSO_4 \cdot H_2O$ contributed to the effect. The fineness of grind was also an important factor.

Our present study indicates that several salt mixtures commonly used in guinea pig studies have a pronounced effect on thiamine stability. An improved salt mixture that was developed is also described.

EXPERIMENTAL

Animals and their care. Guinea pigs (*Cavia porcellus*) of mixed strains and sexes, 6 to 9 days old, and with an average initial weight of about 120 g, were obtained commercially [5] and used throughout this study. For the first 3 days (after receipt of the animals and before starting the experiments), all animals were placed in groups of 3 or 4 and were fed the basal diet supplemented with 2 mg of thiamine·HCl/kg of diet in double-size cages with 0.6-cm mesh floors. During this period the animals were hand-fed and watered twice daily to facilitate their learning to use the hanging watering device and diet cups. The animals, which appeared thrifty and had gained well during the initial 3-day pretrial period, were then allotted to uniform groups according to weight and sex and housed individually in metal cages (24 × 18 × 18 cm) and fed the experimental diets ad libitum for the 5-week trial period. After the animals reached 200 g in weight, the floor mesh-size was increased to 1.3 cm. Cages were cleaned and sterilized once weekly or more often if necessary. Weights were recorded 3 times weekly for each animal and at this time a thorough examination for deficiency symptoms was made. Fresh diet was provided daily and diet cups were changed on alternate days to assure a fresh, clean supply of feed at all times. The laboratory temperature was maintained at 24° ± 1° as a precautionary measure, as Kline et al. (12) have reported environmental temperature to affect the thiamine requirement of rats. Carcass weight [6] was determined on all animals in experiment 2.

[5] Supplied by Dependable Animal Supply Company, Martinez, California.

[6] Carcass weight = weight of animal without entire gastrointestinal tract.

TABLE 1

Composition of basal diet (GPD7) and salt mixture (GPS1)

Basal diet		Salt mixture	
	g/kg diet		*g/90 g*
Casein (vitamin-free)	300	$CaHPO_4$	34.920
Cornstarch	200	K acetate	24.930
Cellulose [1]	150	KCl	7.740
Sucrose (powdered)	100	$CaCO_3$	5.940
Salt mixture (GPS1) [2]	90	NaCl	5.760
Glucose hydrate [3]	72	MgO	4.960
Corn oil	60	$MgSO_4$	4.590
B-vitamin mix in glucose [4]	10	Fe citrate	0.640
Fat-soluble vitamins in corn oil [5]	10	$MnSO_4 \cdot H_2O$	0.370
		KIO_3	0.015
Arginine·HCl	2	$CuSO_4$	0.005
Ascorbic acid	2	$ZnCO_3$	0.130
Choline chloride	2		
Inositol	2	Total (used in 1 kg of diet)	90.00
Total	1,000		

[1] Cellophane Spangles, Rayon Processing Company, Pawtucket, Rhode Island.

[2] New salt mixture formulated for test diets using casein. In experiment 2 the salt mixture was fed at a level of 60 g/kg of diet (and 30 g more of glucose was used).

[3] Cerelose, Corn Products Company, San Francisco.

[4] The following amounts were present per kg of diet: (in milligrams) thiamine·HCl, variable; riboflavin, 16; pyridoxine·HCl, 16; Ca pantothenate, 40; niacin, 200; biotin, 0.5; folic acid, 10.0; and vitamin B_{12}, 0.05.

[5] The following levels of fat-soluble vitamins were present per kg of diet: (in milligrams) vitamin A acetate, 6; α-tocopheryl acetate, 20; vitamin D_3, 0.04; and menadione, 2.

TABLE 2

Growth of guinea pigs fed different salt mixtures in diet GPD7 and retention of thiamine [1,2]

Salt mixture	Amount in diet	No. animals	6-week weight [3]	Thiamine retention (after 4 weeks at 4°)
	%		g	%
GPS1	6.0	12	276± 9.2	94.7
GPS1	9.0	11	250±10.4	89.0
UCB1R [4]	5.25	5	238±25.8	70.2
UCB2R [4]	4.95	11	255± 9.1	85.7
HMW (18)	6.0	11	246± 8.7	67.0
Jones-Foster (19)	6.0	5	234±12.4	56.3
Fox-Briggs (16)	6.0	6	183±17.6	57.1
Stock ration [5]	—	12	280± 8.3	—

[1] All diets contained 16 mg/kg of thiamine·HCl. K acetate (2.5%), MgO (0.5%), and $ZnCO_3$ (0.013%) were added to all diets except those containing GPS1.
[2] Summary of 2 trials. No animals died during these experiments.
[3] Groups averaged 117 g at the start; weights show standard error at 6 weeks.
[4] Unpublished minor modifications of a rat salt mixture described by Briggs, G. M., and M. A. Williams. Federation Proc., 22: 261, 1963. (See thesis mentioned in footnote 7 of the text for detailed composition.)
[5] See footnote 8 of text.

Experimental diets. The basal diet (GPD7, table 1) is a modification of the Reid and Briggs (13) guinea pig diet.[7] Thiamine (as thiamine·HCl) was added at various supplementary levels shown on the tables. New diets were prepared every 2 weeks and stored at 4° in covered glass jars. A commercial stock diet [8] was used as a control in each experiment and was ground to approximately the same degree of fineness as the basal diet.

A new salt mixture (GPS1) was prepared to minimize destruction of thiamine in the purified diet (see table 1). Individual ingredients were chosen which were known to assist in overall stability of dietary ingredients, including thiamine (8–11, 14–16) at levels approximating the guinea pig's requirement for various minerals as far as known (including the minerals present in a casein diet). Potassium acetate and a source of magnesium were included in the salt mixture to avoid the more common practice of adding them separately. In experiment 1 the salt mixture was fed at a 9% level and at a 6% level in experiment 2.[9]

Diets which differed only in the kind or level of salt mixture were prepared to compare in guinea pigs (in a 6-week test) and to check the effect of various salt mixtures on thiamine stability in diets in storage (details in table 2). In these studies the level of thiamine was kept constant (16 mg thiamine·HCl/kg diet). Thiamine determinations were made by the thiochrome method of the Association of Vitamin Chemists (17), omitting the enzymatic digestion and column purification steps. Samples were run in triplicate assays.

RESULTS

The effect of different salt mixtures on growth and on thiamine stability in storage of the diets under refrigerated conditions is shown in table 2. The new mixture (GPS1) provided satisfactory growth and appeared to be less destructive to thiamine at both the 6% and 9% levels than any of the other salt mixes tested except possibly UCB2R (designed for rat diets) which was added at a lower level than most of the other salt formulations. The Hubbell et al. (18) and the Jones-Foster (19) salt mixtures (both designed for rat diets) and the Fox-Briggs (16) salt mixture (for chickens) were the most destructive of all under these conditions, with thiamine retentions of 67, 56, and 57%, respectively.

The growth and survival data of the guinea pigs in experiment 1, fed various levels of thiamine·HCl (table 3), showed optimal survival with 1.7 mg thiamine·HCl/kg diet,

[7] Details of diet formulation, mixing procedures, and growth curves of these animals may be found in the M.S. degree thesis of Mrs. Katherine C. Liu, titled, "Thiamine Requirement of the Guinea Pig and the Effect of Salt Mixtures on Thiamine Stability," 1965, on file in the Library of the University of California, Berkeley.
[8] Purina Guinea Pig Chow, Ralston Purina Company, St. Louis.
[9] We now add 7.5% of the salt mixture to casein diets for routine purposes.

TABLE 3

Experiment 1 (summary of 2 trials): [1] *Growth response of guinea pigs fed graded levels of thiamine·HCl in diet GPD7 with 9% salt mixture*

Thiamine·HCl	No. of animals		5-week gain
	Initial	Final	
mg/kg of diet			*g*
0.2	7	0	—
1.0	13	7	66±20.7 [2]
1.3	6	4	97±17.4
1.7	6	6	181±12.2
2.0	13	13	173±14.9
2.3	13	13	211± 8.7
2.7	6	6	218± 3.8
3.0	12	12	209± 9.9
8.0	13	13	217± 9.7
Stock [3]	12	12	232± 7.8

[1] Average initial weight was 123 g for all groups, consisting of 6 to 7 guinea pigs each.
[2] Mean ± SE.
[3] See footnote 8 of text.

but maximal growth rate was not reached until 2.3 mg of thiamine·HCl was included/kg of diet. In experiment 2 (table 4) optimal survival was reached with 1.7 mg as with the higher salt level, but maximal growth was observed with 2.0 mg thiamine·HCl/kg diet. The level of supplementation also had a profound effect upon the percentage of carcass weight of live weight of survivors indicating maximal growth, as measured by this criterion as well, was reached at 2 mg of thiamine·HCl/kg of diet.

In both experiments 1 and 2 the deficiency symptoms as well as the time of onset of the symptoms were similar. The symptoms observed (many of which were reported by Reid (4, 7)) were: reduced feed consumption and weight gain, rough hair coat, enlarged abdomen, watery eyes, diarrhea, slobbering, and, in extreme cases, head retraction, arched back, hyperextended hind legs, and death. When severely deficient animals were rotated they developed prolonged nystagmus and regained equilibrium with difficulty. The first signs of deficiency were usually noted during the second or third week of the test, and within about one week thereafter, death resulted in the severely deficient animals. The animals that received suboptimal thiamine levels developed fewer symptoms such as a rough-appearing hair coat, a slight distention of the abdomen, and slow growth. When the surviving animals were autopsied it was noted that the ceca in animals receiving 1.7 mg of thiamine·HCl or less were substantially enlarged and had probably contributed to the paunchy appearance. No gross abnormalities were evident at autopsy other than this.

DISCUSSION

This study suggests that salt mixtures are a primary cause of thiamine instability in experimental guinea pig diets, which explains what has appeared to be in the past an exceptionally high requirement for thiamine by this animal (7).

A new salt mixture, which was present at 6% or 9% of the diet, was clearly one of the least destructive to thiamine of any of the salt mixtures tested. Despite the use of the new salt mixture, there is probably still some loss of thiamine·HCl in the diet from other causes (such as heat, other dietary ingredients, fineness of grind, and humidity). In a small exploratory experi-

TABLE 4

Experiment 2 (summary of 2 trials): [1] *Growth response of guinea pigs fed graded levels of thiamine·HCl in diet GPD7 with 6% salt mixture*

Thiamine·HCl	No. of animals		5-week gain	Carcass wt
	Initial	Final		
mg/kg of diet			*g*	*% of live wt*
1.33	8	3	86±34.8 [2]	60.6
1.67	8	7	169±13.0	63.7
2.00	15	15	216± 9.6	69.9
2.33	15	15	208± 8.1	69.0
2.67	8	8	196±11.8	68.7
8.00	15	14	217± 8.1	69.4
Stock [3]	8	8	227± 8.5	70.1

[1] Average initial weight was 114 g for all groups, consisting of 7 or 8 animals each.
[2] Mean ± SE.
[3] See footnote 8 of text.

ment we made, the thiamine content was determined in samples of our diet containing the GPS1 salt mixture left at room temperature for 2 days (approximating what could happen in an animal room). It was found that, under these conditions, when thiamine·HCl was added at a level of 1 mg/kg of diet there was only 55% retention after 2 days, at 2 mg/kg there was 65% retention, and at 8 mg/kg there was 85% retention. Even at zero time (thiamine being assayed as soon as possible after mixing the diet) there were significant losses up to 16% with low levels of thiamine. In this study we have made no attempt to multiply the minimum effective level by some arbitrary factor of loss in order to obtain a true physiological requirement of thiamine, but obviously this might be done or else some other means might be used to supply thiamine if a true requirement is to be obtained (the vitamin might be given by separate supplement, by stomach tube, or by injection).

It can be concluded that the minimum requirement of the growing guinea pig for dietary thiamine is in the range of 2.0 to 2.3 mg/kg of diet, or less. The true physiological requirement for the guinea pig, assuming no loss in the diet, could not be determined under our conditions but appears to be 2.0 mg/kg of diet, or less. Because of the instability of thiamine·HCl in semipurified diets, at least 8 to 16 mg/kg of diet should be added for routine purposes, or a more stable form should be used. Also, a salt mixture should be used which destroys as little thiamine as possible. It can probably be concluded that any unusually high thiamine requirement for a laboratory animal should be looked upon with suspect until the stability of the vitamin in the diet is checked.

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Metabolism of *cis*-11,*cis*-14- and *trans*-11,*trans*-14-Eicosadienoic Acids in the Rat [1]

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ABSTRACT The metabolism of the *cis,cis* and the *trans,trans* isomers of 11,14-eicosadienoic acids was studied with essential fatty acid-deficient adult male rats of the Sprague-Dawley strain. Analysis of liver phospholipids showed that whereas the *trans,trans*-diene isomer was catabolized quickly, the *cis,cis* isomer built up in the liver and was converted to arachidonic acid. The conversion of both the *cis,cis*- and the *trans,trans*-20:2 dienes to 18:2 dienes was demonstrated by isolation and structural analyses of the latter compounds. The conversion of the 20:2 *cis,cis*-diene isomer to linoleic acid and it, in turn, to arachidonic acid was demonstrated by isotope experiments in which methyl *cis*-11,*cis*-14-eicosadienoate-3-^{14}C was injected intraperitoneally into EFA-deficient rats. The retention of 20:2 *cis,cis*-diene appeared to occur as a result of the animal's ability to conserve its stores of EFA. As a facet of this mechanism, the catabolism of the 20:2 *cis,cis*-diene appeared to be arrested with the formation of linoleic acid which was preferentially converted to arachidonic acid.

One of the most intriguing aspects of the interconversion of polyunsaturated fatty acids is the ability of the rat to preserve its store of essential fatty acids, particularly arachidonic acid (1–5). Generally, once the chain of events in the β-oxidation of essential fatty acids is started, it goes to completion (6, 7). However, the possibility that higher polyunsaturated fatty acids may be degraded via the reverse of reactions involved in their biosynthesis has been suggested by Klenk et al. (8, 9). Although it has been pointed out by Mead (6) and Sprecher (10) that simple reversal of these reactions appears remote because the normal intermediate has the α-β double bond in the *trans* configuration (11), several recent reports lend substance to the idea that essential fatty acids may not follow the normal catabolic process. Recently, in this laboratory (12), nutritional studies showed that 4,7,10,13,16-docosapentaenoate gave an increase in liver arachidonic acid of essential fatty acid (EFA)-deficient rats to which it was fed. Schlenk et al. (13) confirmed this conversion with radioactive docosapentaenoate and designated it by the term "retroconversion." Similar reactions have been reported by Nugteren (11) and Sprecher et al. (10).

In the present study both *trans*-11,*trans*-14- and *cis*-11,*cis*-14-eicosadienoic acids (t,t-20:2 and c,c-20:2) gave rise to analogous octadecadienoic acids in the livers of rats and the *cis,cis* isomer was converted to arachidonic acid, at least partially via linoleic acid.

EXPERIMENTAL

MATERIALS AND METHODS

Methyl *cis*-11,*cis*-14-eicosadienoate-3-^{14}C was prepared by chain elongation of linoleic acid-1-^{14}C.[2] The original preparation had a specific activity of 3.6 mCi/mmole. This preparation was methylated with diazomethane (14) and purified by silver-ion thin-layer chromatography (TLC). The purified methyl linoleate-1-^{14}C was reduced to the corresponding alcohol with lithium aluminum hydride and reacted with methanesulfonyl chloride as described by Carroll (15). The methane sulfonate derivative was reacted directly with diethyl malonate-2-sodium in benzene in a sealed ampule in a boiling water bath for 2.5 hours. The crude malonate derivative was saponified and the substituted malonic acid decarboxylated. The decarboxylation was carried out by heating the sample

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[2] Obtained from Tracerlab, Inc., Waltham, Massachusetts.

slowly under nitrogen to 185°; the heating was continued at this temperature until there was no further evolution of carbon dioxide. After the product was cooled, it was esterified with diazomethane (14), recovered and purified by silicic acid-TLC. The final product had a specific activity of 2.75 mCi/mmole and was obtained in a yield of 44.7%. Radio-GLC analysis by the manual technique described by Dutton et al. (16) showed that the final product was >99% 20:2 and of the same degree of radiochemical purity.

Methyl c,c- and t,t-20:2 were prepared via chain elongation by a similar procedure starting with 99% linoleate and linoelaidate, respectively, obtained from the Lipids Preparation Laboratory of The Hormel Institute. Gas-liquid chromatography (GLC) showed that these esters were more than 99% pure.

Gas-liquid chromatography (GLC). The analysis of methyl esters was carried out with an F & M Model 1609 hydrogen flame gas chromatograph with a 240-cm× 0.62-cm column packed with 10% ethylene glycol succinate phase [3] on 100–120 mesh Gas Chrom P. The analyses were carried out at 185° with a carrier gas flow rate (nitrogen) of 65 ml/minute. Detector response with the 16- through 20-carbon chain saturated methyl esters was very nearly linear on standard mixtures and thus fatty acid composition was calculated directly from the proportionalities of the peak areas.

Preparative gas chromatography was carried out with an F & M Model 500 gas chromatograph equipped with a thermal conductivity detector. The isolations were carried out with a 240-cm × 0.62-cm column packed with 20% by weight of diethylene glycol adipate polyester phosphoric acid on 60–80 mesh chromosorb W [4] at 175° with a helium flow of 60 ml/minute. The samples were collected by condensing them in a gradient cooled glass tube that extended under the surface of a small amount of chloroform in a test tube or, in the case of radioactive esters, by the manual collection system described by Dutton et al. (16) using 15 ml of scintillation solution in a vial for counting.

Determination of structure and isomer composition of fractions of unsaturated fatty acids were determined by reductive ozonolysis by the method of Nickell and Privett (17).

Experiment 1. Adult male rats of the Sprague-Dawley strain, of 200- to 250-g weight, were fed ad libitum for 10 months a fat-free diet consisting of vitamin-free test casein (24.5%), cellulose [5] (4.0%), minerals [6] (4.0%), sucrose (66.5%) and 1% vitamin mix (18).[7] The growth of the animals leveled off at an average of 419 g, and all animals exhibited mild dermal symptoms of essential fatty acid deficiency. The c,c- and t,t-20:2 were fed to groups of 4 and 3 animals, respectively, at a level of 5% by weight of the diet for 11 days. The former animals consumed approximately 55 g, and the latter approximately 38 g of the fatty supplements. The animals were fasted for 12 hours at the end of the supplementation period and killed by withdrawal of blood from their aortas after they were placed under light ether anesthesia. The major organs of the animals were excised, frozen on dry ice, and stored at −20° before extraction of the lipids. Five animals that received only the fat-free diet were also killed and the organs excised in a similar manner.

The livers of the animals in each group were combined and extracted 3 times with chloroform-methanol (2:1, v/v) in a ratio of 10 volumes of solvent to 1 g of tissue in a Virtis homogenizer. The filtered solutions were evaporated to near dryness and the lipid extracted from the residues with freshly distilled chloroform. The phospholipid fraction was isolated and purified by acetone precipitation at 5°. The fractionation was monitored by silicic acid-TLC to assure complete separation of the neutral lipids and to keep the loss of phospholipids to a minimum.

The phospholipid fractions were then interesterified by heating them in sealed

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[3] EGSS-X, Applied Science Laboratories, State College, Pennsylvania.

[4] Lachat Chemicals, Inc., Chicago.

[5] Non-nutritive cellulose, Alphacel, Nutritional Biochemicals Corporation, Cleveland.

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[7] Vitamin mix, B-mix 75. Obtained from General Biochemicals, Inc.

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ampules with an excess of 6% HCl in dry methanol under an atmosphere of nitrogen in a boiling water bath for 2 hours. The esters were recovered by usual procedures and analyzed by GLC. The 18:2, 20:3 and 20:4 esters were isolated by a combination of silver-ion TLC and preparative gas-liquid chromatography and their structures determined via ozonolysis.

Experiment 2. Adult male rats of the Sprague-Dawley strain with EFA deficiency, as used in the first experiment, were placed under light ether anesthesia and injected intraperitoneally with 0.5 to 5 μCi of methyl *cis*-11,*cis*-14-eicosadienoate-3-^{14}C. The radioactive ester was injected as an emulsion with bile salts and physiological saline prepared in a minimum volume in the bottom of a conical centrifuge tube. The amount of activity injected into the animals was determined by measurement of the amount of radioactivity placed in the tube and that remaining in the tube and syringe after each injection. Four animals were injected and killed by exsanguination as described above at intervals of 1, 2, 4 and 6 hours. The major organs were excised and frozen on dry ice. Livers were extracted as in experiment 1 and the amounts of radioactivity incorporated into the lipids were determined by counting aliquots in scintillation solution in a Packard TriCarb scintillation spectrometer. Representative aliquots were interesterified as in experiment 1 and the percentage distribution of the radioactivity among the liver fatty acids was determined by counting the activity of the individual methyl esters isolated by preparative GLC using the manual technique described by Dutton (16). Averages of duplicate 10-minute counts were obtained by liquid scintillation counting. When necessary to obtain counts at least twice that of background multiple collections of samples were made in the preparative GLC.

RESULTS AND DISCUSSION

The fatty acid analysis of the phospholipids isolated from the livers of the animals in the first experiment are presented in table 1. Since the animals used in this experiment had not been fed a fat-free diet until they had reached a weight of 200 to 250 g, the arachidonic acid content of the liver phospholipids was still fairly high, even after 10 months of a fat-free diet. However, the 20:3 content of the liver phospholipids was elevated, as was the ratio of the triene to tetraene in accordance with the characteristics of an essential fatty acid deficiency (19).

TABLE 1
Liver phospholipid analysis

	Fat-free group	*trans,trans*-20:2 group	*cis,cis*-20:2 group
18:2	2.9	5.6	7.8
20:2	0.4	0.4	8.5
20:3	13.0	10.9	8.8
20:4	9.5	8.7	18.8

A relatively high level of the fatty ester supplements was fed for a short period in the present study instead of either a low level for a prolonged period or less to a larger number of animals because Schlenk et al. (20) and Rahm et al. (21) demonstrated that the former type of feeding gave the greatest and quickest response to changes in the fatty acid composition of the liver lipids. In fact, Lyman et al.[8] reported recently that the normal levels of arachidonic and linoleic acids could be restored and the triene-to-tetraene ratio reduced to normal in EFA-deficient rats by the forced feeding of a single large dose of safflower seed oil. In the latter study the fatty acid picture returned to normal in a period of 19 hours after the dose was administered.

The analyses in table 1 show that whereas c,c-20:2, which is a member of the linoleic acid family, accumulated in the liver phospholipids, the t,t-isomer was apparently catabolized. This observation is in accord with the work reported by Coots et al. (22) showing that the 18-carbon *trans*-dienoic acid isomers were catabolized more quickly than linoleic acid and supports the contention generally held that the stores of essential fatty acids are conserved by the animal. In accordance with the general effects of essential fatty acids (19), the phospholipids of the animals receiving the c,c-20:2 contained less 20:3 and more 20:4 than the animals that received the t,t-20:2 or the animals maintained with the fat-free diet.

Infrared and structural analyses of the 20:3 and 20:4 fractions isolated from the

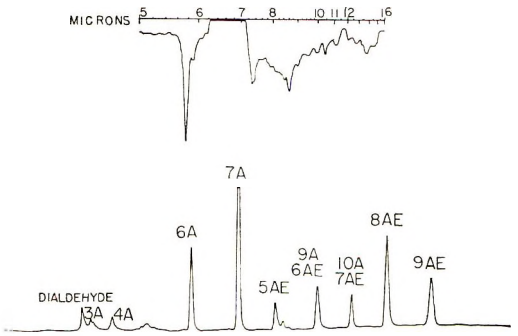

Fig. 1 Infrared spectrum (upper curve) and structure via ozonolysis (lower curve) of the 18:2 fraction isolated from t,t-20:2-fed animals. *A* denotes aldehyde; *AE* denotes aldester; the number before the letter denotes the chain length of the fragment.

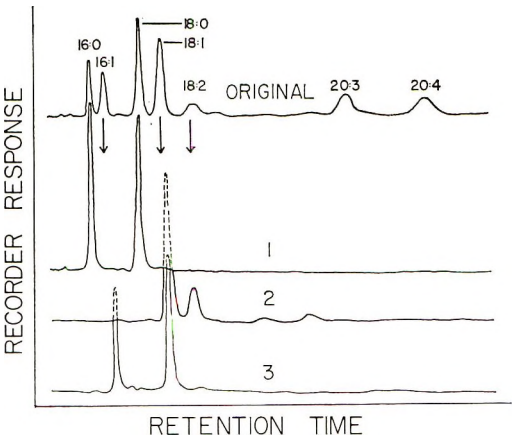

Fig. 2 GLC analyses of fractions separated by silver-ion TLC of the kidney phospholipids (as methyl esters) of the t,t-20:2-fed animals. Nos. 1, 2 and 3 represent the analysis of bands received from the plate beginning at the top.

animals fed t,t-20:2 showed that little of it was converted to higher polyunsaturated fatty acids. The relatively large amounts of 18:2 in the phospholipids indicated that these acids were a major product of the metabolism of 20:2. To demonstrate this point, the 18:2 fraction from the animals fed t,t-20:2 was isolated by preparative GLC and its composition determined by infrared spectral analysis and ozonolysis (fig. 1). Figure 1 demonstrates the presence of *trans,trans*-linoleic acid in this fraction, the expected product of the β-oxidation of the t,t-20:2, by the band for *trans* unsaturation at 10.3 μ and the presence of hexanal (6A) and azelaldehydate (9AE) showing the presence of a diene with double bonds in the 9,12-position. Other fragments (fig. 1) showed that the fraction also contained diene members of the palmitoleic and oleic families of acids normally prevalent in EFA-deficient animals.

Further evidence for the conversion of t,t-20:2 to t,t-18:2 was obtained by analysis of the phospholipids of the kidney isolated and converted to methyl esters in similar manner as the liver phospholipids. The presence of t,t-18:2 in this case was demonstrated by a combination of silver-ion TLC and GLC (fig. 2). Figure 2 shows that a monoene band (band no. 2) contained 18:2. Only dienes with *trans,trans* double bonds migrate with *cis*-monoenes in silver-ion TLC (23). That the diene in this band did not represent contamination from a lower band was demonstrated by analysis of the next lower band (band no. 3, fig. 2, lower part of band no. 2) which also contained only monoenes 18:1 and 16:1.

The purpose of the radioactive experiment was to confirm that c,c-18:2 was formed from c,c-20:2, in accordance with the observed increase of 18:2 in the liver lipids found in the first experiment (table 1).

The amounts of radioactivity injected intraperitoneally and the amounts recovered in the liver lipid over the various time periods are shown in table 2. The portion of the total radioactivity found in the liver under these conditions appeared to increase through the first 4 hours and either to reach a plateau or a peak sometime between the fourth and sixth hours. It was assumed that the test fatty acid

TABLE 2

Intraperitoneal injection of methyl eicosadienoate-3-^{14}C and recovery of radioactivity in liver lipids

	Hours after injection			
	1	2	4	6
Injected, dpm × 1000	10278	9255	4092	1070
Recovered, dpm × 1000	164	364	276	75
Recovered, %	1.6	3.9	6.7	7.0

was being absorbed by the liver, metabolized and removed during all of these time-periods and the radioactivity not accounted for in the lipid was assumed to have been as yet unabsorbed, distributed to other tissues, or expired as $^{14}CO_2$.

The changes in the percent distribution of the radioactivity in the total liver fatty acids, the object of the experiment, are summarized in table 3. These results show the pattern of interconversion of the family of essential fatty acids, and support the nutritional findings of experiment 1.

It is apparent from these results (table 3) that c,c-20:2 is converted in part to linoleic acid. The radioactive 20:3 could have been produced from the conversion of the fed 20:2, directly, as well as 18:2 derived from 20:2 via 18:3, or 20:2 in accordance with the pathways shown in figure 3. Since c,c-11,14-20:2 is only about one-half as active as linoleic acid in curing an EFA deficiency (24), the major pathway for its conversion to arachidonic acid may be via linoleic acid (fig. 3). If the major pathway were via the 20:3, it could be expected to be at least as active as linoleic acid. Also significant in this respect are the recent in vitro enzymatic studies of Mohrhauer et al. (25), which suggest that linoleic acid is converted to arachidonic acid more efficiently via the 18:3 triene than by the 20:2 diene. That radioactive 18:3 was found in the present study indicated that linoleic acid was being formed from the fed 20:2 diene and converted to arachidonic acid via this pathway (fig. 3).

TABLE 3

Distribution of radioactivity in liver fatty acids at various time intervals after injection of methyl eicosadienoate-3-^{14}C

	Hours after injection			
	1	2	4	6
	% of total cpm			
16:0 + 16:1	16.2	8.2	15.3	17.9
18:0	2.4	2.8	2.7	3.5
18:1	1.2	2.7	4.2	3.0
18:2	4.0	9.3	9.9	14.2
18:3	3.0	3.6	4.2	3.5
20:2	60.6	48.0	31.5	35.9
20:3	5.2	14.9	20.0	10.3
20:4	2.5	3.6	5.7	5.1
Unidentified long-chain acids	4.8	5.0	6.3	6.6

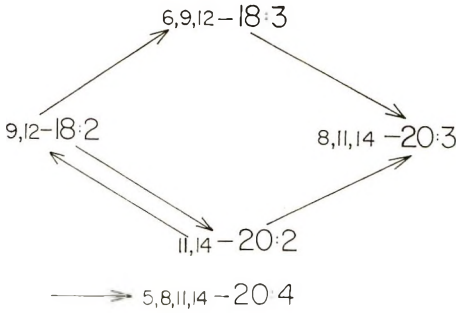

Fig. 3 Pathways in the interconversion of the linoleic family of acids.

In view of the observation that a single large dose or diet high in linoleic acid can quickly restore the liver stores of EFA,[9] and that the normal levels of EFA are not increased greatly beyond normal values by feeding very high levels of linoleic acid (26), it appears that the animal has a mechanism for the control of the metabolism of essential fatty acids. Conservation of the stores of EFA in time of stress, such as on the prolonged feeding of a fat-free diet, as evidenced by the slow depletion of arachidonic acid in the livers of adult animals maintained with a fat-free diet, appears also to reflect the existence of a mechanism for the regulation of the metabolism of EFA. Although there was some oxidation of c,c-20:2 beyond the 18-carbon stage, as evidenced by a scattering of radioactivity in lower unsaturated and saturated fatty acids (table 3), its oxidation was largely arrested at the initial metabolite of the EFA family of acids, namely, linoleic acid, and converted to arachidonic acid, apparently as a facet of the conservation process. Possibly, the enzymes involved in catabolism of fatty acids cannot compete as strongly for linoleic acid as those involved in its synthesis to arachidonic acid. Since t,t-unsaturated fatty acids are not converted readily to higher polyunsaturated fatty acids, apparently there is no interference in their catabolism.

ACKNOWLEDGMENT

The authors are very grateful to J. D. Nadenicek for his interest and advice in

[9] See footnote 8.

the application of chain elongation techniques for the preparation of the compounds used in this study.

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Catabolism of ^{14}C-Labeled Thiamine by the Rat as Influenced by Dietary Intake and Body Thiamine Stores [1]

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ABSTRACT Whether the rate of thiamine catabolism is influenced by the extent of body stores was investigated. Three groups of weanling rats were injected with 8, 40, or 100 μg ^{14}C-thiazole thiamine until their stores were considered to be in equilibrium with intake. The excretion of urinary thiamine (with *Lactobacillus viridescens*), urinary thiamine metabolites (radioactivity) and $^{14}CO_2$ was measured. The magnitude of the excretion of $^{14}CO_2$ correlated well both with thiamine intake and thiamine levels in liver, kidney and muscle. No correlation, however, was observed with brain thiamine levels. The excretion of thiamine metabolites in urine showed an excellent correlation with intake but urinary thiamine per se was a poor index of intake. The excretion of urinary radioactivity after a single injection of radioactive thiamine gave some insight into body stores but under these conditions, $^{14}CO_2$ was not detected. Because a stoichiometric relationship between caloric intake and thiamine metabolism was not observed, it is suggested that the destruction of thiamine does not occur primarily during its participation in energy-yielding reactions.

In a previous study, (1) the generation of $^{14}CO_2$ from ^{14}C-thiazole-labeled thiamine by the rat appeared to be independent of intake and of body thiamine stores. Thus, the possibility existed that $^{14}CO_2$ production might serve as a measure of endogenous thiamine metabolism. Because the experimental conditions in our first study were such that the exact status of the body stores was not known, further studies have now been carried out and are reported in this publication.

EXPERIMENTAL PROCEDURE

Thirty weanling male rats of the Sprague-Dawley strain were housed in groups and fed a thiamine-deficient diet (2) for 2 weeks, after which time 4 rats were killed for the determination of baseline tissue thiamine levels (muscle, liver, kidney, brain). The remaining rats were then assembled at random into 3 groups of 8, 8 and 10 rats. All groups were continued with the ad libitum feeding of the thiamine-free diet and water and were injected intraperitoneally daily with 8, 40 or 100 μg of ^{14}C-thiazole-labeled thiamine,[3] respectively. During this experimental period, the rats were housed in pairs in stainless steel metabolism cages constructed to permit separate collection of urine and feces.

Urine samples were collected daily in glass bottles containing 1 ml of 2 N acetic acid, pooled by group and frozen before analysis. Aliquots of each urine pool were counted daily in a liquid scintillation counter to determine radioactivity. Pooled weekly urine samples from each group were analyzed for thiamine activity with *Lactobacillus viridescens*. After the rats had been on their experimental regimens for 4 weeks, collections of respiratory CO_2 were begun. One rat from each group was placed in a respiration chamber and CO_2 was collected for 24 hours by drawing the expired air through two successive chambers each of which contained 300 ml of a mixture of ethanolamine and ethylene glycol monoethyl ether (1:3 by volume) (3). At the end of the 24-hour period, the two trapping solutions were pooled, mixed and duplicate samples were taken for scintillation counting. On completion of the CO_2

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[3] Specific activity, 5,600 cpm/μg.

collection, the rat was killed and his tissues taken for thiamine analysis. In preparation for analysis, the tissues were steamed with 0.1 N HCl for 30 minutes and then treated with Mylase [4] to convert the phosphorylated forms of thiamine to free thiamine. The latter were determined microbiologically using *L. viridescens* (4).

RESULTS AND DISCUSSION

The group growth responses to the 3 levels of thiamine injection are shown in figure 1. It should be borne in mind that the group sizes were being diminished by one each week after experimental day 30. The 40- and 100-μg intake levels both supported equivalent and good growth but a reduced growth rate was noted at the 8-μg intake level. This suboptimal growth, also obtained in a previous study (5), was not unexpected because the minimum requirement for growth of the rat has been stated to be 12.5 μg/day (6).

The thiamine concentration in liver, muscle, kidney and brain obtained during the course of the experiment is shown in figure 2. Each point represents a value for a single rat and the rats were injected with their respective doses of thiamine for 30 days before the collection of data was commenced. It is presumed that this period of time was adequate for equilibration of thiamine stores with thiamine intake.

The liver thiamine concentration of the 3 groups segregated clearly. The approximate means were 7, 4 and 1 μg/g at the 100, 40- and 8-μg levels of intake, respectively. Liver thiamine concentration declined in all groups as the experiment progressed even though the 40- and 100-μg levels of intake are far in excess of the needs for growth. The marked apparent increase the last week of the experiment in the rat receiving 100 μg thiamine/day is unexplained.

Kidney thiamine concentrations were also found to be a function of intake but they did not decrease with the passage of

[4] Mann Research Laboratories, New York 10006.

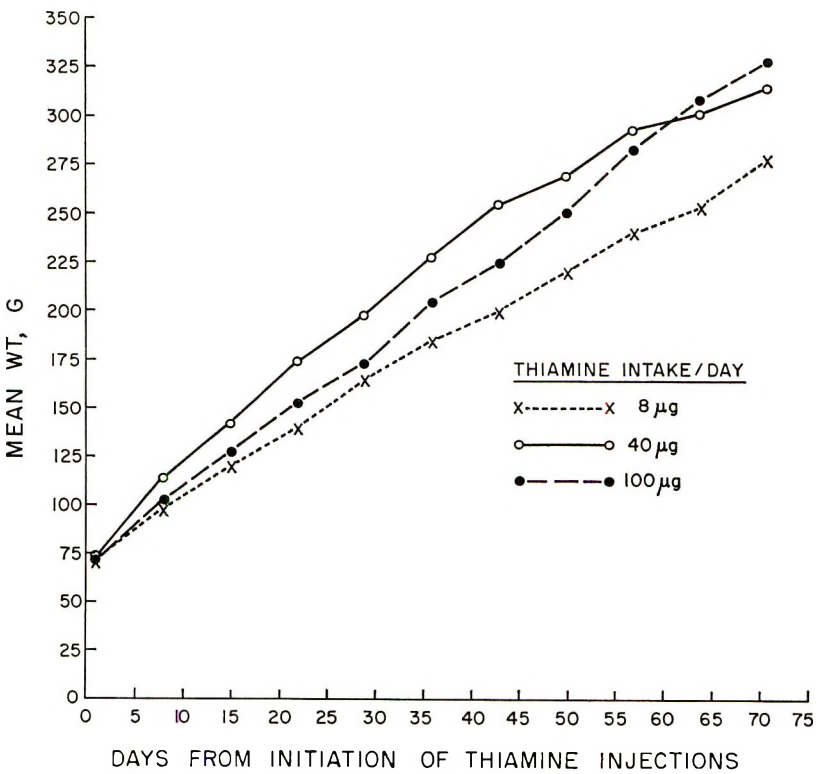

Fig. 1 Growth response of rats to different levels of thiamine intake.

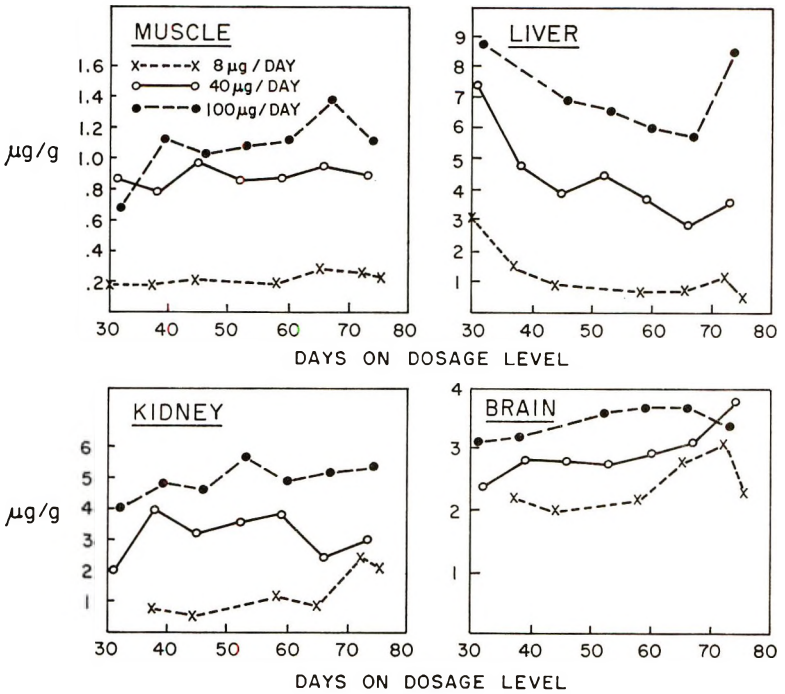

Fig. 2 Variation of mean tissue levels of thiamine with time at 3 levels of intake. Each point represents a value from a single rat. Thiamine was determined by *L. viridescens* assay.

time. In fact, if anything, the tendency was for a slight increase. The same could also be said for muscle thiamine concentration, which also increased somewhat with time. The muscle thiamine content at the 8-µg intake level was remarkably low and quite invariable.

Although brain thiamine concentration reflected the differences in thiamine intake, the marked differences found in other tissues were not observed. For example, the differences between the 100- and 8-µg levels of intake in thiamine concentration in livers, kidney and muscle were approximately 7-, 5- and 5-fold, respectively. This difference did not reach twofold in the brain. This is further evidence for the ability of this organ to maintain thiamine stores under adverse conditions.

Figure 3 records the micrograms of thiamine "excreted via the lung" as calculated from the radioactivity of the collected $^{14}CO_2$ assuming the specific activity of tissue thiamine was the same as that injected. The amount of thiamine destroyed as measured by $^{14}CO_2$ excretion showed a positive correlation with the amount of thiamine injected. This is in direct contrast with our earlier findings in which it was observed that the excretion of $^{14}CO_2$ was relatively constant at 3 levels of thiamine intake (30, 50, 100 µg/day). It is now obvious that the tissue thiamine stores in this early study were not markedly different.

The relationship between thiamine destroyed as estimated from $^{14}CO_2$ excretion and thiamine levels in liver, kidney, muscle and brain is shown in figure 4. A remarkably good correlation was observed in muscle, liver, and kidney but no meaningful relationship was observed with brain thiamine level. The influences of various dietary and physiological factors remain to be determined but the potential use of the measurement of $^{14}CO_2$ excretion for nondestructive estimates of body thiamine stores in the rat is evident. Unfortunately, the requirement that the body thiamine stores be uniformly labeled with ^{14}C-thiazole-labeled thiamine precludes use of this approach in the human.

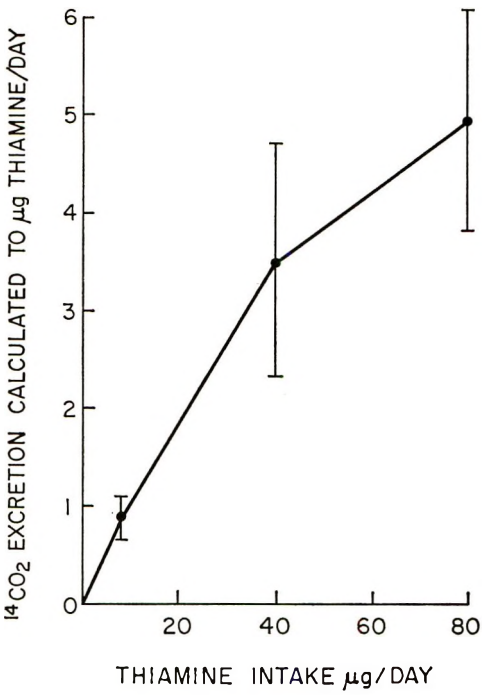

Fig. 3 Mean 24-hour $^{14}CO_2$ excretions expressed as micrograms of thiamine at 3 levels of thiamine intake. Each point represents the mean excretion of 8 rats. Vertical bars represent one standard deviation.

The failure of urinary thiamine excretion levels to relate linearly to body stores has been long suspected and is impressively illustrated by the data presented in figure 5. Urinary thiamine (*L. viridescens*) activity does not permit one to distinguish between those rats injected with 8 μg/day and those injected with 40 μg/day. Increasing the intake 2.5-fold (40 to 100 μg/day) resulted in a 17-fold increase in the urinary excretion of thiamine.

The relationship between urinary thiamine metabolite excretion (excluding thiamine) and intake, on the other hand, is remarkably linear (fig. 5). Thus, it is very likely that the measurement of thiamine metabolites in urine rather than thiamine per se would be eminently satisfactory in the assessment of thiamine status in the human. Unfortunately, these compounds must be identified and quantitative techniques set up for their determination before they can be so used.

An attempt to estimate approximate thiamine balance in the 3 groups of rats is seen in table 1. All data shown were determined in this study except for the estimated fecal loss of thiamine. Earlier studies with ^{14}C-pyrimidine-labeled thiamine (5) and with ^{35}S-labeled thiamine (1) suggested fecal losses of about 15% at several levels of intake. More recently, studies with ^{14}C-thiazole-labeled thiamine suggest losses of up to 25% of intake. We have used the mean of these 2 figures (20%) for approximating fecal losses in these calculations.

The approach to balance is rather good considering the difficulties in making quantitative collections of urine and feces, the arbitrary use of a single value for estimation of fecal thiamine loss, and the fact that the data were collected during a period of growth. Possible errors introduced by coprophagy are probably insignificant in comparison with those just

TABLE 1

Thiamine balance in rats at three levels of thiamine intake

Daily intake	Thiamine [1] in urine	Thiamine [2] metabolites in urine	Thiamine [3] excreted via lung	Estimated [4] fecal loss	Total intake accounted for
μg	*μg/day*	*μg/day*	*μg/day*	*μg/day*	*%*
8	0.1	4.3	0.8	1.6	85
40	1.2	20.5	3.4	8.0	83
100	17.0	55.9	4.9	20	98

[1] *L. viridescens* assay. Values represent the means of data obtained on 10 weekly urine pools of each group.
[2] Radioactivity of urine converted to micrograms of thiamine and corrected for urinary thiamine by *L. viridescens* assay. These data represent the means of approximately 70 daily measurements of urinary radioactivity in pooled urine samples of each group.
[3] $^{14}CO_2$ converted to micrograms of thiamine. These data represent the means of a single value obtained on each of 8 rats during the course of the experiment.
[4] Calculated as being 20% of thiamine intake.

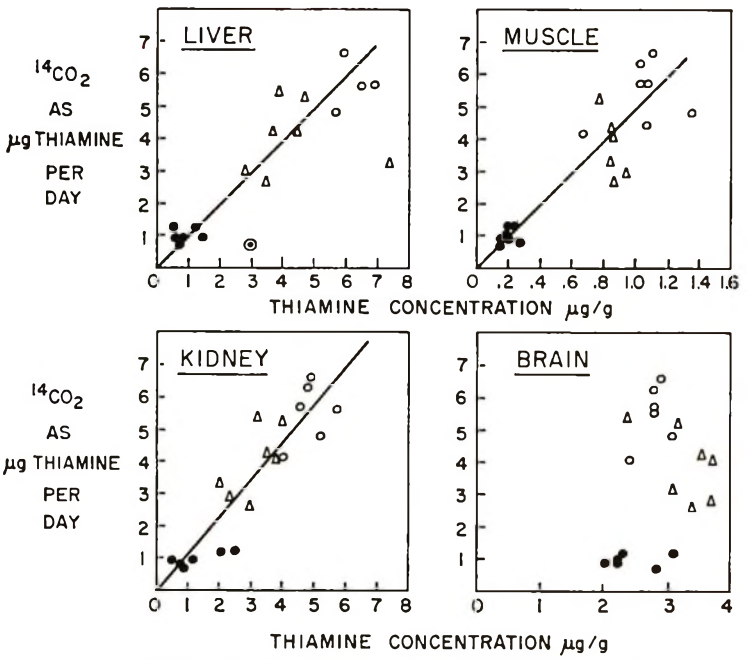

Fig. 4 Mean 24-hour $^{14}CO_2$ excretions expressed as micrograms of thiamine as a function of tissue thiamine levels determined by *L. viridescens* assay. Each point represents data for a single rat on a specific thiamine intake. Intake legend: 8 μg/day ●; 40 μg/day△; 100 μg/day ○.

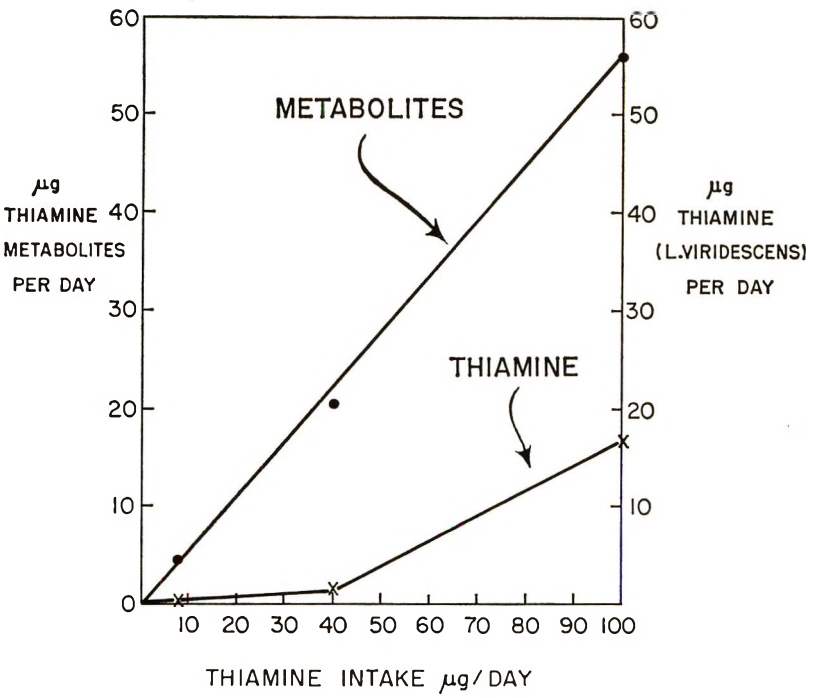

Fig. 5 Dietary thiamine intake and the excretion of thiamine and its metabolites. Metabolite data are means of approximately 70 daily measurements of urinary radioactivity in pooled group urine samples corrected for the presence of thiamine. Thiamine data represent the means of data obtained on 10 weekly urinary pools of each group (*L. viridescens* assay).

listed. Virtual balance was attained at the 8-μg level of thiamine intake with an apparent retention of about 1 μg of thiamine/day/rat. At the 40-μg level of intake the apparent retention was 7 μg; at the 100-μg intake level, about 2 μg were retained. The "crudeness" of our data does not permit great significance to be attached to these particular numbers but it is clear that thiamine balance can be attained at several levels of intake. This has been suspected, of course, for many years, but as far as the authors are aware it has not been demonstrated until now. Presumably at thiamine levels too low to permit growth, the rats would be in negative balance — excreting more thiamine and metabolites than consumed.

That the excretion of $^{14}CO_2$ responds rapidly to changes in tissue stores is shown in figure 6. In this study, 2 rats that had received 100 μg thiamine daily for 75 days were maintained with the thiamine-deficient diet but without further injections of thiamine. A 24-hour collection of CO_2 was made every other day during the 33-day experimental period and the urinary excretion of radioactivity was determined daily. On this regimen the decline in expired radioactivity with time is less abrupt than that observed in urine. This might be expected because, in dietary deprivation the excretion of thiamine is rapidly suppressed which results in an initial steep decline in the excretion of radioactivity. Although we do not have data showing changes in tissue thiamine levels, it is probable that the reduction in $^{14}CO_2$ excretion faithfully follows the decrease of tissue thiamine stores.

Although the amount of $^{14}CO_2$ excreted is a function of the concentration of thiamine in its principal body depots, practical use of this observation for the assessment of thiamine status under ordinary conditions is not possible. It was of interest, therefore, to determine whether the excretion of the radioactivity of a single dose of ^{14}C-thiamine might be related to body stores. Adult Sprague-Dawley rats which had previously received no radioactive thiamine, were given single injections of 100

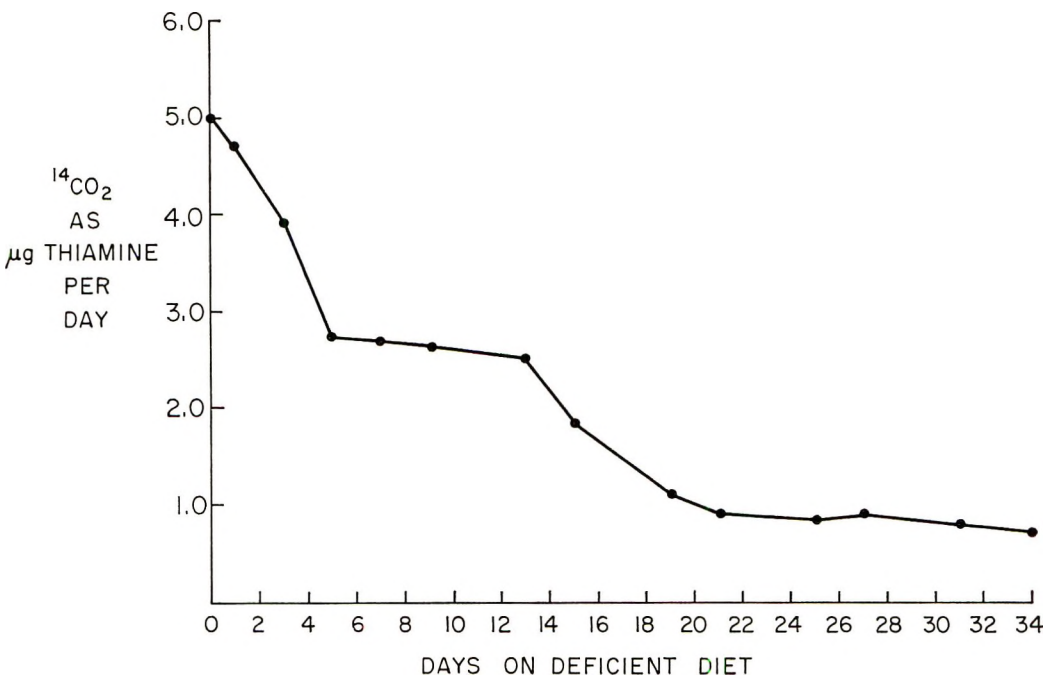

Fig. 6 $^{14}CO_2$ excretions calculated as thiamine in 2 rats fed a thiamine-deficient diet after having received daily injections of 100 μg of ^{14}C-thiazole thiamine for 90 days. Each point represents the mean excretions of the 2 rats on that particular day.

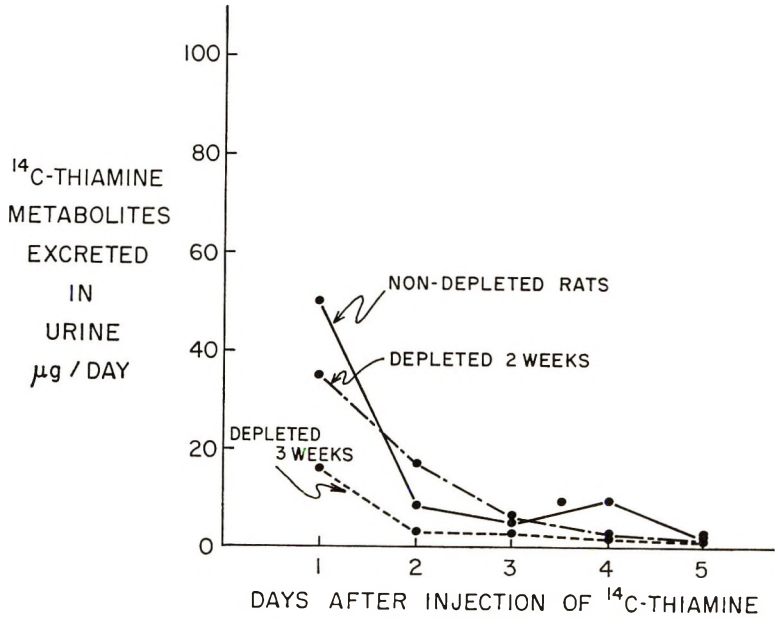

Fig. 7 Daily urinary radiometabolite excretions of adult rat pairs on different dietary regimens after receiving a single injection of 100 μg ^{14}C-thiazole thiamine.

μg of ^{14}C-thiazole-labeled thiamine. Carbon dioxide and urine collections were carried out for a 5-day period. No detectable radioactivity in the expired CO_2 could be found in any of the 5 consecutive daily collections. The same experiment was then repeated on pairs of adult rats that were fed a thiamine-deficient diet for one week and two weeks, respectively, before the single injection of ^{14}C-thiazole labeled thiamine. Again, no radioactivity was detected in the expired CO_2.

The daily urinary excretion of radioactivity in these pairs of rats is shown in figure 7. The proportion of radioactivity that appeared daily in the urine after the injection was an inverse function of the length of time the animals had consumed the depleted diets. Thus, these data suggest that the excretion of thiamine and its metabolites after a load test may reflect body stores.

CONCLUSIONS

These studies demonstrate clearly that the rate of $^{14}CO_2$ generation by rats uniformly labeled with ^{14}C-thiazole thiamine depends principally upon the magnitude of body thiamine stores. Thus, our previous data which suggested that the amount of $^{14}CO_2$ generated daily from ^{14}C-thiazole-labeled thiamine was an index of endogenous thiamine turnover proved to be erroneous. When thiamine intakes and tissue levels are high, balance is maintained by enhanced destruction and also by the increased excretion of urinary thiamine.

Because the groups receiving 40 and 100 μg thiamine/day grew at similar rates, it may be presumed that they had similar calorie intakes. Despite this, the rats receiving 100 μg of thiamine produced more metabolic products of thiamine — both in urine and as $^{14}CO_2$. Thus, there is no stoichiometric relationship between calories used and thiamine destroyed, at least at relatively high levels of thiamine intake. This conclusion has further implications as to the cellular site of the destruction of thiamine. It appears that thiamine may be catabolized only slightly during its participation in the Krebs cycle and the hexose-monophosphate shunt with the bulk of its catabolism being carried out by enzymes which may or may not be specific. Thiaminase activity, well-known to be present in several species of fish and bacteria, has recently been detected in the tissues

of the rat [5] and it is probable that this enzyme(s) is responsible for the bulk of thiamine catabolism.

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Production of Zinc Deficiency in the Squirrel Monkey (*Saimiri sciureus*) [1]

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ABSTRACT Zinc deficiency was produced in the squirrel monkey by feeding a low zinc diet containing casein as the protein source. The casein was rendered low in zinc by a procedure combining isoelectric precipitation and treatment with disodium ethylenediaminetetraacetic acid (Na_2 EDTA). The diet was tested in two experimental trials, in which eleven, weanling, male squirrel monkeys were fed the diet for periods up to 352 days and were compared with eleven control monkeys fed the same diet supplemented with 15 μg zinc/g. Growth in monkeys fed the low zinc diet was retarded; the animals became unkempt in appearance, and some showed varying degrees of hair loss. Hair samples obtained on days 22–23 of feeding showed decreased zinc concentrations. Red cell volumes and red cell hemoglobin concentrations, determined serially until days 56–57, were normal. At killing, reduced zinc concentrations were found in the heart, spleen, liver, and pancreas. In the adrenal, zinc concentration was increased, while in bone it was normal. Three monkeys fed the low zinc diet for more than 130 days showed decreased concentrations of serum albumin and zinc.

The zinc requirement of experimental animals has been the subject of numerous investigations. Its essentiality for rats (1), swine (2), and birds(3,4) is established but no previous attempts have been made to induce zinc deficiency in the primate. This is of particular importance because it has been recently suggested that spontaneous zinc deficiency in the human occurs in the Middle East (5,6). The present report describes the production of zinc deficiency in the squirrel monkey (*Saimiri sciureus*). This species was chosen for study because it is small, easy to handle, and has a low food consumption.

EXPERIMENTAL

The purified diet detailed in table 1 was patterned after that used by Dutra de Oliveira et al. (7) in studies with the rhesus monkey. For the present study, casein was treated to reduce its zinc content and additional trace amounts of selenium, cobalt, and molybdenum were added. Also, higher levels of vitamins were used. The zinc content of the purified diet varied somewhat depending upon the batch of casein used but did not exceed 0.5 ppm.

The procedure for the preparation of zinc-low casein was as follows: 9 kg of purified casein [4] were suspended in approximately 180 liters of distilled water in a cylindrical Polyethylene jar and brought into solution by the addition of concentrated NH_4OH (approximately 675 ml); 90 g of sodium ethylenediaminetetraacetic acid (Na_2EDTA) were added and the solution was stirred for about 15 minutes; the casein was then reprecipitated by the slow addition (with stirring) of 6 N HCl until the pH was approximately 4.5; the precipitate was washed 3 to 4 times with 180-liter volumes of deionized water; and the supernatant solution was removed by using 4000-ml glass beakers. The dissolving of casein with NH_4OH, stirring with Na_2EDTA, precipitation with HCl and washing of the precipitate was repeated.

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[2] Taken in part from a thesis submitted in partial fulfillment of the requirements for the Ph.D. at Vanderbilt University.

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[4] Nutritional Biochemicals Corporation, Cleveland.

TABLE 1
Composition of zinc-low basal diet

	g/100 g
Casein [1]	18.0
Cottonseed oil [2]	10.0
Mineral mix (modified HMW) [3]	3.82
Vitamin mix [4]	2.0
Choline chloride	0.2
Vitamin C	0.1
Sucrose	65.88

[1] Casein was rendered low in zinc by a procedure combining isoelectric precipitation and chelation with ethylenediaminetetraacetic acid (EDTA) (see text for details of this procedure).
[2] Wesson Oil, Wesson Oil Sales Company, Fullerton, California; to this was added vitamin A, 3250 IU (Aquasol A, U. S. Vitamin and Pharmaceutical Corp., New York); vitamin D_2, 650 IU (Drisdol, Winthrop Laboratories, New York); and α-tocopherol, 20 mg.
[3] Hubbell et al. (9); the following salts were also added: (in micrograms) $CoCl_2 \cdot 6H_2O$, 32; $Na_2SeO_3 \cdot 5H_2O$, 27; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 7.4.
[4] Each 2 g contained: (in milligrams) thiamine·HCl, 1.5; riboflavin, 1.5; pyridoxine·HCl, 1.5; Ca pantothenate, 9.0; biotin, 0.02; folic acid, 0.5; vitamin B_{12}, 0.0025; niacinamide, 22.5; inositol, 20; menadione, 10; and sucrose to make 2.0 g.

The washed casein was collected and strained through cotton gauze to remove excess water. The product was spread in a thin layer on trays lined with Parafilm and dried at 60°. The dried casein was powdered by grinding in a Waring Blendor. This procedure gives a yield of from 65 to 75% of the original weight of the casein. By analysis, the zinc content of different batches of the final product was from 1.3 μg/g to 2.7 μg/g. The casein sample before treatment contained 25 μg/g of zinc. One batch of vitamin-free casein [5] contained higher levels of zinc (39 μg/g). Long-term chronic studies on the effects of EDTA added to the diets of rats have shown that the compound is non-toxic (8) and we consider that the final product contained inconsequential amounts of this compound. If present, this chelating agent might be expected to enhance zinc availability rather than to decrease it (9).

The HMW salt mixture (10) was prepared from individual salts. Different batches of reagent grade commercial salts required for preparation of the mixture were analyzed individually for zinc by atomic absorption spectrophotometry. Zinc concentrations varied from none to 8.8 μg/g. Hence the mineral mixture for these studies was prepared using salts found to contain the lowest amounts of zinc. To the final HMW mixture, supplemental salts (see table 1) were added. Commercial sucrose, cottonseed oil and vitamins, presumed to be essentially free of zinc, were used without further purification.

Young male squirrel monkeys,[6] weighing approximately 300 g were housed in cages constructed locally of Plexiglas.[7,8] Each cage consisted of two housing units separated by a removable middle partition. Diets and deionized water were dispensed ad libitum in plastic cups. To minimize food spillage, the monkeys were trained to consume in a short time their rations to which 10 ml deionized water had been added to each 90 g diet immediately before feeding. Initially, food was offered 3 times a day for 1 to 2 hours. When the animals learned to accept the diet, it was offered twice daily for 15 to 30 minutes. In instances when a monkey did not adjust to the diet readily, the food was left in the cage for longer periods. Food intakes were recorded daily, and the animals were weighed weekly in the morning before feeding. In the first experiment, the monkeys were kept in a room maintained at 22° and 45% relative humidity, in the second, the temperature was raised to 27° at which temperature the monkeys were more active. On termination of the experiment the monkeys were killed with an overdose of sodium pentobarbital and the major organs were removed and immediately weighed. Portions were preserved for histological studies and for zinc analyses. The samples for zinc analyses were weighed immediately and frozen.

For zinc analyses the tissues were digested in a mixture containing 17 parts concentrated reagent grade nitric acid and 3 parts 70% perchloric acid (v/v).[9] Digestions were carried out in 30-ml Kjeldahl flasks and approximately 4 to 5 ml of digestion mixture was used for each sample weighing up to 0.5 g. Each digest was diluted with deionized water to a concentration suitable for analyses and then analyzed for zinc in a Perkin Elmer Model 303 atomic absorption spectrophometer. A

[5] See footnote 4.
[6] Tarpon Zoo, Tarpon Springs, Florida or the Pet Farm, Miami, Florida.
[7] Owens-Corning Fiberglass. Toledo, Ohio.
[8] Economy Plastics, Nashville, Tennessee.
[9] The procedure for the analysis of zinc in plant materials was used as quoted in Analytical Methods for Atomic Absorption Spectrometer, Manual no. 990-9461, Perkin-Elmer Corporation, Norwalk, Connecticut, 1964.

standard curve was constructed using solutions containing zero, 1, 2, and 3 ppm zinc. Reagent grade zinc metal dissolved in redistilled 6 N HCl (1 mg/ml) was used as a stock standard.

Total serum proteins were determined by a biuret procedure (11). Serum proteins were fractionated by paper electrophoresis using a Beckman-Spinco Analytrol. Red cell counts were made by using a Model B Coulter Counter.[10]

RESULTS

Preliminary trial - 6 monkeys. In an exploratory study to determine adequacy and acceptability of the diet, 6 male monkeys were fed the low zinc regimen for 23 days. Those animals which accepted the diet immediately, consumed about 25 g/day. Others consumed little diet during the first few days of the study. To approximate the magnitude of the daily zinc losses during the trial period, 24-hour urine samples and feces collections were made on day 14 of the regimen and analyzed for zinc. The daily loss averaged 95 μg of zinc per monkey and this level was used as a rough guideline to determine an appropriate dietary level of zinc.

On day 23, by which time the monkeys readily accepted the diet, they were divided into 2 groups. Three monkeys (nos. 1, 2, and 3) were continued with the low zinc diet and the other three (nos. 4, 5, and 6) were fed the same diet supplemented with 15 ppm zinc (as $ZnSO_4 \cdot 7H_2O$). This provided an additional 375 μg of zinc /25 g of diet—an amount 4 times that of the estimated daily loss. Hemoglobin concentrations at this time ranged from 15 to 17 g/100 ml except for monkey no. 4 which was slightly anemic (11.9 g/100 ml).

Clinical observations—preliminary experiment. Monkey no. 1. This male monkey had difficulty in accepting the diet and was given an oral glucose solution periodically during the first few days. By day 23 its hair was sparse over the lateral thorax, medial sides of the arms and thighs, and over the knees. Ten days later, after being fed the low zinc diet for 33 days, it died. During the last week before death its appetite was erratic and 2 days before death, it had episodes of vomiting and the vomitus was streaked with blood. The animal had lost 53 g, weighing 244 g at death. Because autolysis of tissues prevented use of microscopic diagnostic procedures, the exact cause of death was not determinable. We feel that it was probably due to causes other than zinc deficiency.

Monkey no. 2. This male was the largest and probably the oldest in the group. It was very active and ate well, consuming as much as 80 g of diet/day during the last week of the experiment. It gained 154 g (final weight 629 g) during 352 days with the low zinc diet. At autopsy the skin over the knees was reddened and thickened, but examination of histologic sections revealed no parakeratosis. Although the animal's general appearance was unkempt, its hair looked normal.

Monkey no. 3. There were no gross external changes in this male during the initial feeding of the low zinc diet but its early food consumption of 20 to 25 g /day gradually declined to 10 to 15 g. This was accompanied by slow loss of weight and muscular weakness. The animal became indifferent to the other monkeys and sat in one corner of the cage with its head lowered and resting on its knees. It was killed after being fed the low zinc diet for 105 days. Its hair was sparse over the whole body, the losses were particularly marked over the knees, the dorsal aspects of the feet, hands, and lateral thorax. There were oval, 3- to 4-mm diameter, ulcerated lesions of the skin of the medial aspects of both knees, probably because of the constant apposition of these regions. It had lost 97 g and weighed 256 g when killed. A photograph made 5 days before killing is shown in figure 1.

Monkey no. 4. While fed the low zinc diet, this male monkey was relatively inactive and developed alopecia of the proximal half of its tail. When fed the zinc-supplemented diet, the hair reappeared and it became more active. Its initial hemoglobin level of 11.3 g/100 ml increased to 16.0g/100 ml 18 days after receiving zinc. When killed after being fed the zinc-supplemented diet for 107 days, it had gained 111 g of body weight. Shortly before termination of the experiment it ate from 40 to 50 g of food daily.

[10] Coulter Electronics, Hialeah, Florida.

Fig. 1 Photographs of control monkey no. 6 fed the zinc-supplemented diet (left) and monkey no. 3 fed a low zinc diet. Photographs were made after each monkey had been fed his respective diet for 99 days in the preliminary experiment.

Monkey no. 5. By day 23 of low zinc feeding alopecia had occurred over the lateral portions of the legs and over both shoulder areas of this male monkey. Subsequently it was fed the zinc-supplemented diet for an additional 60 days during which time it gained 79 g and its hair returned to normal.

Monkey no. 6. This male monkey accepted the diet readily. It appeared normal throughout the initial feeding of the low zinc diet and gained 186 g during the 330 days of consuming the zinc-supplemented diet. A photograph taken on the same day as that of monkey no. 3 is shown in figure 1.

Periodic examination of blood smears revealed the presence of flagellates similar in morphology to trypanosomes in all monkeys in this experiment. Monkey no. 4 was more heavily infected, and on one occasion, as many as 12 flagellates were seen in a blood smear.

Second experiment — sixteen monkeys. Based on the experience obtained in the first group, a second more elaborate experiment was designed. Twenty young male squirrel monkeys were fed for 20 days the zinc supplemented diet (15 ppm) used in the preliminary trial. During this period, hematocrits were determined; blood smears were examined for red cell morphology and the presence of parasites, and fecal smears were examined for parasitic ova. There was no radiologic evidence of pulmonary or abdominal lesions. Four monkeys were discarded because they had low hematocrit values or showed marked anisocytosis, or gained little or no weight during the 20 days fed the zinc-supplemented diet. The 16 remaining monkeys who had gained an average of 46 g each,

were divided into 2 groups and housed individually. Eight animals (monkeys 1a–8a) having a mean weight of 315 g (range 252–352 g) were fed the low zinc diet (<0.5 ppm). The other eight (monkeys 1b–4b and 1c–4c) having a mean weight of 323 g (range 284 to 343 g) were continued with the zinc-supplemented diet. The

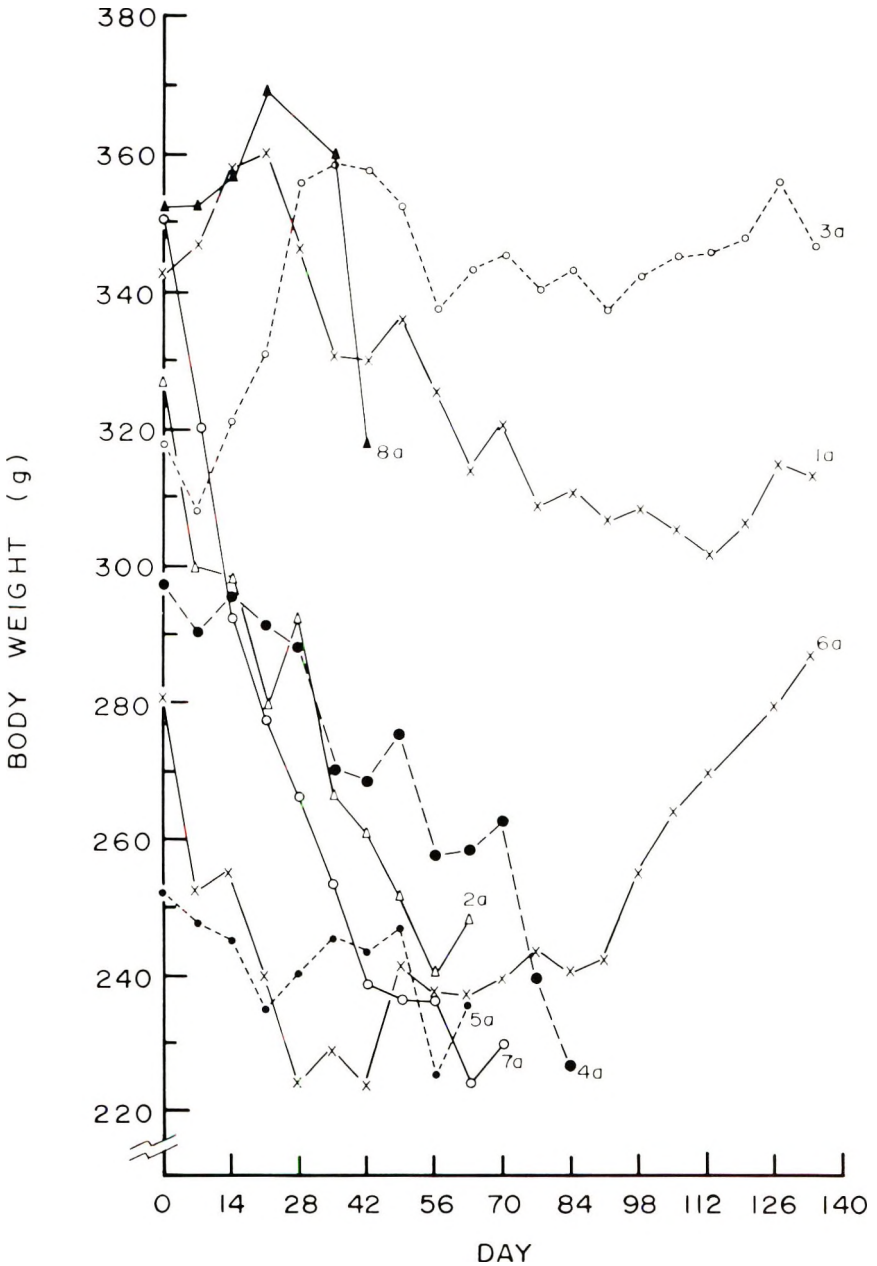

Fig. 2 Growth curves of monkeys fed the low zinc diet during the second experiment. The numbers represent the individual monkeys involved. These monkeys were fed the zinc supplemented diet for 20 days before day zero at which time they were transferred to the low zinc diet.

feeding trial was considered to start at this point, that is, day 1 of the experiment coincides with the transfer of monkeys to the low zinc diet.

The growth curves of individual animals are shown in figure 2 ("zinc-deficient") and figure 3 ("zinc-supplemented"). A summary of individual weight changes and weights of the livers and spleens on termination of the experiment is shown in table 2. Parasitological data obtained from fecal analyses, gross inspection of the intestinal tract at killing, microscopic examination of blood smears and histologic

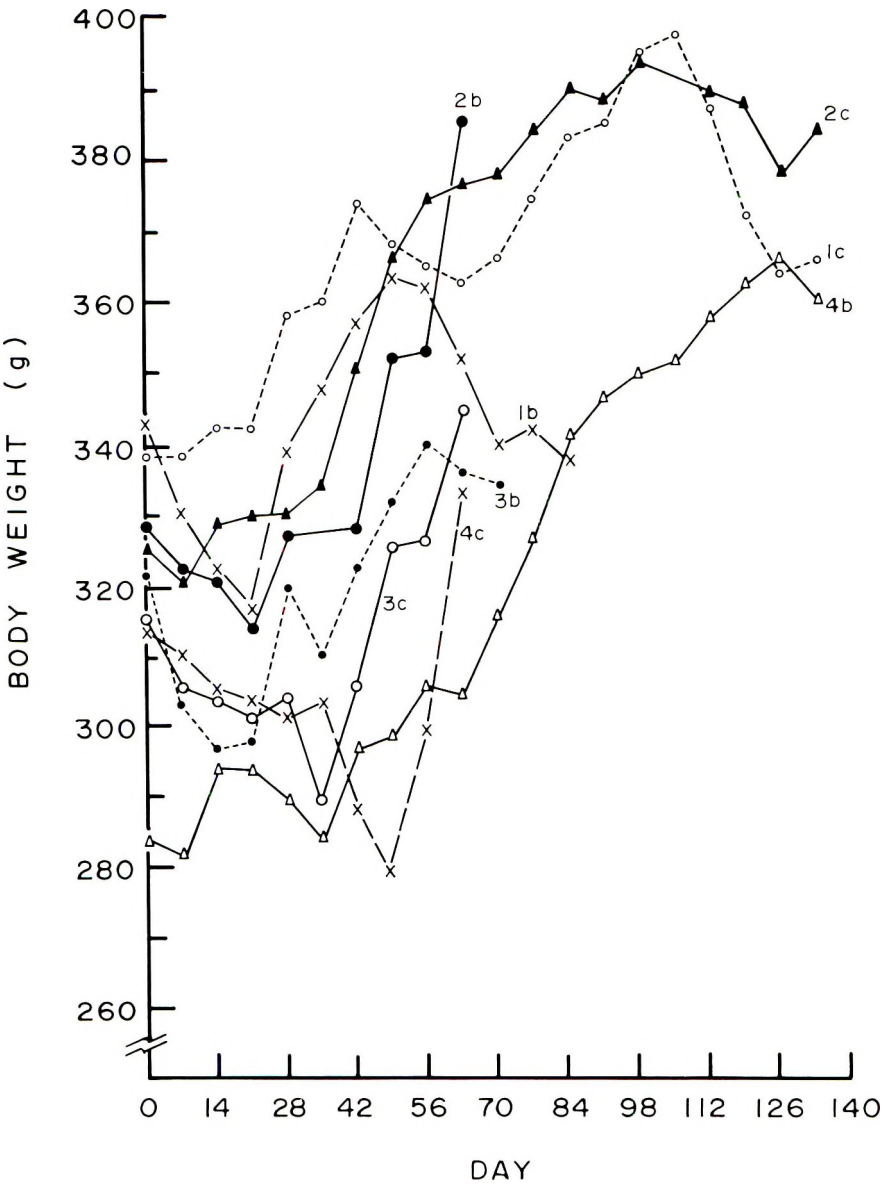

Fig. 3 Growth curves of monkeys fed the zinc-supplemented diet during the second experiment. The numbers represent the individual monkeys involved. These monkeys were fed the zinc-supplemented diet for 20 days before initiation of these growth measurements which commenced on day zero.

TABLE 2

Summary of changes in body weights, and weights of the spleens and livers of squirrel monkeys fed a low zinc and zinc-supplemented diet

Monkey no.	Days fed experimental diet	Body wt when killed	Weight change	Spleen wt	Liver wt
		g	*g*	*mg*	*g*
		Low zinc diet			
1a	134	310.5	− 31.5	321	8.82
2a	61	284.3	− 79.1	—	—
3a	135	346.5	+ 28.1	571	9.57
4a	84	225	− 72.0	315	9.39
5a	64	247	− 5.3	309	9.15
6a	136	272.5	− 14.5	757 [1]	9.86
7a	71	231.8	−120.0	220	8.06
8a	40	317.8	− 34.1	780 [1]	18.76 [1]
		Zinc-supplemented diet			
1b	86	338	− 5.0	562	11.66
2b	61	385.2	+ 55.8	—	—
3b	72	334.3	+ 12.5	266	9.27
4b	136	358.5	+ 74.1	732 [1]	11.32
1c	134	366	+ 28.1	535	12.28
2c	134	387	+ 72	414	16.93 [1]
3c	65	346	+ 31.5	357	10.29
4c	63	335	+ 21	652 [1]	10.44

[1] Organs enlarged in comparison with those of other monkeys.

TABLE 3

Parasitologic data of squirrel monkeys (exp. 2)

Monkey no.	Fecal ova [1]	Intestinal lumen [2]	Larvae in lungs [3]	Blood flagellates [4]
1a	8	1 thorny-headed worm	−	−
2a	7	none	+	−
3a	—	none	−	−
4a	—	none	−	−
5a	12	none	−	−
6a	—	none	−	−
7a	—	1 thorny-headed worm	+	−
8a	6	2 tapeworms; 1 roundworm; 1 thorny-headed worm	+	+
1b	5	1 tapeworm	−	+
2b	29	2 thorny-headed worms	−	−
3b	3	none	−	−
4b	9	none	−	+
1c	9	none	−	+
2c	10	none	+	−
3c	2	none	−	−
4c	11	1 tapeworm; 7 thorny-headed worms	+ + +	−

[1] Counts of various ova based on a fecal smear 4 days before start of experiment.
[2] Based on gross inspection of the intestinal tract at the time the animals were killed.
[3] Based on random microscopic sections of lungs; larvae similar in morphology to strongyloides.
[4] Based on a single blood smear; flagellates similar in morphology to trypanosomes.

sections of the lungs are tabulated in table 3.

Clinical observations (exp. 2). Deficient monkeys: Monkey 8a gained 120 g during the initial 20 days on the zinc-supplemented diet, but after 3 weeks of being fed the low zinc diet, its appetite suddenly decreased and it lost weight precipitously. On the morning of day 40 it died (fig. 2). At autopsy, the animal was found to have several intestinal parasites (table 3). Its liver and spleen were markedly enlarged (table 2). Examination of histologic sections of the lungs showed many areas

infiltrated with strongyloides larvae. It is not possible to state the exact cause of death but it is likely that both zinc deficiency and parasitism were involved.

Monkey 7a gained 51 g during the 20 days of being fed the zinc-supplemented diet. When fed the low zinc diet, however, it failed to show further increase in weight. On day 8 of the latter diet, its hemoglobin level was 13.6 g/100 ml and red blood cell count, 5.8 million/mm^3. On day 15, it passed moderate amounts of fresh blood in feces and subsequently the feces were streaked with blood. On day 21 hemoglobin had dropped to 9.7 g/100 ml and the red cell count was 5.2 million/mm^3. The hemoglobin decreased further to 6.4 g/100 ml and the red cells to 4.58 million/mm^3 on day 28. When killed on day 70, the animal was markedly pale and emaciated. Microscopic examination of sections of the small intestines revealed larvae similar in morphology to strongyloides in the intestinal wall. The larvae were surrounded by infiltrates of lymphocytes, plasma cells and eosinophils, indicating a chronic inflammatory reaction. One thorny-headed worm, genus acanthocephala, was found with its head embedded in the wall of the ileocecal junction. The liver and spleen were pale, but were comparable in size to many of those of the other monkeys in this group (table 2).

Monkeys 2a and 4a gained 59 and 49 g, respectively, during the initial 20 days of the zinc-supplemented diet. When transferred to the low zinc diet, they rapidly and progressively lost weight. There was no evidence of parasitism in monkey 4a. Monkeys 5a and 6a, initially the smallest monkeys in this group, also showed a rapid decline in weight. Although no parasites were demonstrated in monkey 6a, its spleen was found to be enlarged on autopsy (table 2).

Control monkeys: The monkeys fed the zinc-supplemented diet (1b–4b; 1c–4c) showed in general, progressive increases in weight. This occurred even though they were no less parasitized than those fed the deficient ration. Monkey 1b, however, had periods of fluctuation in weight and was losing weight when killed on day 85. On autopsy it was found to have a tapeworm. Although monkey 4c had a greater intestinal worm load (a tapeworm and seven thorny-headed worms) and progressively lost weight until day 49, it recovered this loss after anti-helminth treatment (see below) and was gaining weight when killed on day 63.

From days 37 to 40 on the experiment each surviving monkey in both groups was given an oral dose of 1 ml of a suspension containing 25 mg of 2-(4′thiazolyl)-benzimidazole,[11] a drug effective against a number of helminths and larvae in the tissues (12). After this course of treatment, monkey 6a (fig. 2) started gaining weight, but the growth of the other monkeys in the zinc-low group was unaffected. Among the monkeys fed the zinc-supplemented diet (fig. 3) monkey 4c alone appeared to derive benefit from the treatment.

Monkeys 1a and 3a, that were fed the low zinc diet for the longest period, showed hair loss which was marked laterally on both upper arms and on the thorax and abdomen. The other deficient monkeys in this series showed no marked abnormality in their hair coats, but their unkempt appearance made them easily distinguishable from those fed the zinc-supplemented diet. Gross lesions of the skin were not seen.

Hematology. Periodic determinations on hematocrit, hemoglobin, and red cell counts from day 8 to 29 showed no significant differences between the animals fed the low zinc and zinc-supplemented diets (table 4). Slightly higher values for hematocrit and red cell counts were obtained in the zinc-supplemented group on days 56–57, but the mean corpuscular volumes and corpuscular hemoglobin concentrations were comparable. Values from monkey 7a, which had a gross rectal hemorrhage, are excluded from table 4.

Serum proteins and zinc concentrations. Total serum proteins, albumin and globulin fractions obtained from heart blood samples of monkeys killed after they had been fed the experimental diets for more than 130 days are shown in table 5. The mean albumin level is slightly but significantly lower ($P < 0.05$) in the monkeys fed the low zinc diet. The difference between the mean γ-globulin levels was not statistically significant. Serum zinc concentrations, however, were markedly and

[11] Thiabenzole, Merck and Company, Rahway, N. J.

TABLE 4

Mean hematocrits, hemoglobin concentrations, red cell counts, mean corpuscular volumes (MCV) and mean corpuscular hemoglobin concentrations (MCHC) in squirrel monkeys fed a low zinc and zinc-supplemented diet

Days fed experimental diet	Hematocrit	Hemoglobin	RBC	MCV	MCHC
	%	*g/100 ml*	*million/mm³*	*μ³*	%
			Low zinc [1]		
8–9	44.4 ± 1.3 [2]	14.7 ± 0.5	6.25 ± 1.14	71.9 ± 3.8	23.8 ± 1.2
21–22	47.2 ± 1.2	15.5 ± 0.5	6.69 ± 1.85	70.7 ± 2.1	23.1 ± 0.7
28–29	48.1 ± 1.6	15.9 ± 0.7	7.28 ± 0.62	66.1 ± 1.2	21.8 ± 0.7
56–57	48.1 ± 0.9	16.6 ± 0.4	7.80 ± 0.16	65.7 ± 1.5	21.3 ± 0.5
			Zinc-supplemented [3]		
8–9	44.2 ± 1.7	14.4 ± 0.7	7.05 ± 0.41	63.7 ± 3.1	20.7 ± 1.0
21–22	44.3 ± 0.9	14.6 ± 0.6	6.24 ± 0.31	71.9 ± 3.4	23.6 ± 0.9
28–29	48.5 ± 0.8	15.8 ± 0.2	7.46 ± 0.70	65.5 ± 1.7	21.3 ± 0.8
56–57	51.6 ± 0.9	17.0 ± 0.5	8.18 ± 0.20	64.0 ± 1.1	21.1 ± 0.6

[1] Data from 6 animals; those from monkey 7a were excluded; and no hematologic data were obtained from monkey 8a because of a 1-cm stump of scar at the tip of the tail.
[2] Values are means ± 1 SE.
[3] Data from 8 animals.

TABLE 5

Serum zinc and protein concentrations of squirrel monkeys fed a low zinc and zinc-supplemented diet

Monkey no.	Days fed diet	Total proteins	Albumins	α-globulins	β-globulins	γ-globulins	Albumin / globulin	Zinc
		g/100 ml	*g/100 ml*	*g/100 ml*	*g/100 ml*	*g/100 ml*		*μg/100 ml*
				Low zinc diet				
1a	130	6.62	3.74	1.13	0.68	1.07	1.30	73.6
3a	135	6.57	3.73	1.00	0.74	1.10	1.31	87.9
6a	136	6.62	3.84	0.86	0.59	1.33	1.38	64.5
Mean		6.60	3.77 [1]	1.00	0.67	1.17	1.33	75.3 [1]
				Zinc-supplemented diet				
4b	136	6.46	4.11	0.89	0.60	0.86	1.75	107.0
1c	134	7.04	4.20	1.17	0.69	0.98	1.48	106.0
2c	134	6.34	3.96	0.87	0.72	0.79	1.66	101.3
Mean		6.61	4.09	0.98	0.67	0.88	1.63	104.8

[1] Significantly different from mean of zinc-supplemented group ($P < 0.05$).

uniformly lower in the monkeys fed the low zinc diet ($P < 0.05$).

Tissue zinc concentrations. The zinc concentrations of different organs from 9 squirrel monkeys fed the low zinc diet and from 11 monkeys fed the zinc-supplemented diet from both experiments were analyzed and the results are summarized in table 6. The heart, spleen, liver, pancreas and hair of the monkeys fed the low zinc diet showed significantly lower zinc concentrations but the testes did not. Unexpectedly, the concentrations of zinc in their adrenals were higher. The mean weight of these glands per 100 g body weight in the zinc-deprived monkeys was 51.6 mg, which is slightly higher than the mean of 43.1 mg of those of the zinc-supplemented monkeys. Thus, the higher adrenal zinc concentrations in the former group were not associated with reduced adrenal size.

Among the monkeys fed the low zinc diet, monkey 7a had the lowest liver zinc concentration (14.1 μg/g). It is probable that the repeated hemorrhages in this monkey enhanced the loss of tissue zinc. Of the other animals, the liver of monkey 3 (exp. 1) had the lowest zinc level (21.9 μg/g) which is comparable to its external

TABLE 6

Comparison of tissue zinc concentrations of squirrel monkeys fed a low zinc and zinc-supplemented diet [1]

Organ	Zinc concn			P value
	Low zinc diet (A)	Zinc supplemented diet (B)	Difference (B − A)	
	μg/g wet wt	*μg/g wet wt*	*μg/g wet wt*	
Esophagus	18.9 ± 2.3(9) [2]	19.9 ± 0.6(11)	− 1.0	ns [3]
Stomach	19.6 ± 0.6(9)	22.1 ± 1.4(11)	− 2.5	ns
Duodenum	18.0 ± 1.1(8)	20.2 ± 1.2(11)	− 2.2	ns
Muscle	28.5 ± 2.4(9)	24.0 ± 3.1(11)	+ 4.5	ns
Heart	18.9 ± 0.8(8)	22.0 ± 0.9(11)	− 3.9	< 0.05
Lung	16.0 ± 1.3(8)	19.1 ± 1.1(11)	− 3.1	ns
Spleen	18.7 ± 0.8(9)	21.3 ± 0.5(11)	− 2.6	< 0.05
Liver	37.8 ± 4.1(9)	51.4 ± 3.6(10)	−13.6	< 0.05
Pancreas	32.8 ± 2.8(9)	48.1 ± 5.0(11)	−15.3	< 0.05
Kidney	22.8 ± 1.8(8)	28.7 ± 2.6(11)	− 5.9	ns
Testes	18.4 ± 3.3(6)	17.0 ± 0.6(10)	+ 1.4	ns
Adrenal	24.6 ± 2.8(8)	15.7 ± 1.1(10)	+ 8.9	< 0.005
Femur	72.4 ± 3.1(9)	79.8 ± 3.1(11)	− 7.4	ns
Parietal bone	91.2 ± 4.7(9)	96.0 ± 3.4(11)	− 4.8	ns
Hair [4]	184.0 ± 3.3(8)	241.0 ± 27.9(8)	−57.0	< 0.005

[1] Values are ± 1 SE.
[2] Numbers in parentheses indicate number of samples.
[3] Not statistically significant ($P > 0.05$).
[4] Samples obtained 22–23 days after start of second experiment.

appearance of severe deficiency. Monkey no. 1 (first experiment) that had been fed the low zinc diet for only 33 days showed the highest value (56.1 μg/g).

The histopathologic changes observed in the zinc-deficient monkeys are described in an accompanying publication (13). The most characteristic histopathologic feature was parakeratosis of the tongue.

DISCUSSION

It is clear from the growth data that zinc is a required nutrient for the young squirrel monkey. Growth failure and an unkempt appearance accompanied occasionally by hair loss were the most typical growth signs of deficiency. No hematologic changes or drastic alterations in serum protein patterns were detected other than a slight depression in the serum albumin level.

These data do not permit more than a crude estimate to be made of the zinc requirement of the squirrel monkey but they do provide information useful in planning a practical dietary intake. A diet containing approximately 15 ppm of zinc supported good growth, whereas growth failure resulted when a diet containing approximately 0.5 ppm was fed. The failure to include a zinc salt in the mineral mixture added to a diet containing commercially available casein (either "purified" or "vitamin-free") would yield a diet containing about 4 to 5 ppm of zinc. Whether this would support adequate growth is not known. A daily intake of about 1 μg zinc /g of body weight appears to be a reasonable value to use in the planning of casein-based diets for studies with *S. sciureus*. Based on experience with the rat, it is likely that the zinc requirement of the monkey would be considerably higher if fed a diet containing either soybean protein or excess calcium, or both. Such diets reduce the availability of zinc (14).

The lower zinc content of the hair in the deficient monkeys is of interest inasmuch as the zinc content of hair has been proposed as an index of body zinc stores (15). This observed difference may not be related necessarily to progressive zinc depletion. Rather, it is possible that the difference results from accumulation of zinc in the hair of the controls as observed in rats (16). Whatever the cause, the painless availability of hair gives a high priority to studies of its zinc content as influenced by intake and body stores. The finding of a significantly reduced serum zinc level in the monkeys fed the deficient diet

suggests that this may also serve as a measure of zinc body stores.

The following findings in the squirrel monkey differ from those described in zinc-deficient rats (16); anorexia was not prominent except terminally, liver zinc concentrations were decreased while the concentrations of zinc in bone and testes were normal; there were no significant histologic lesions of the esophagus or skin.

In the rat the difference in bone zinc concentration in zinc-deficient and zinc-supplemented animals is due largely to a marked increase in the bone zinc content in rats fed the supplemented diets[12]. Although the failure to observe such a difference in this study might be due to differences in the zinc content of the monkey and rat control diets (15 ppm vs. 30 ppm) it is more likely that the monkey studies were not carried out over a sufficient segment of the life span of the monkey to observe such an effect.

It is characteristic of zinc deficiency in the rat that the zinc concentration in the testes falls rapidly and pathological changes are seen early (16, 17). Thus, it was unexpected to find no significant differences in zinc content or pathological changes in the testes. Again, this may be a function of the length of the present study.

The significantly lower concentration of zinc in the heart, spleen, liver and pancreas implicates these soft tissues as reservoirs of zinc in the monkey. It is not likely that parasitism caused these reduced levels even though Van Peenen and his co-workers (18, 19) reported that human livers with various pathologies including schistosomiasis have a low zinc concentration. It appears improbable, however, that the reduced liver zinc concentration in our deficient monkeys was due to trypanosoma infections. Booth and Schulert (20) have reported recently that the body zinc stores of the golden hamster are not reduced by experimental schistosomiasis infestation and it is possible that schistosomiasis affects zinc stores and metabolism only if it causes the losses of large quantities of blood.

The possibility that the lower zinc concentration in the monkey livers was due to reduced feeding also appears to be unlikely. Chronic anorexia was not characteristic of the deficiency syndrome in the monkey, and Harrison (21) and Macapinlac (16) noted no differences in the zinc content of livers of starved or pair-fed rats, respectively.

The highly significant increase in adrenal zinc concentration in the monkeys fed the low zinc diet deserves comment. The relative weight of these glands has been found to be increased in zinc-deficient male rats (16). Rudzik and Riedel (22, 23) observed variations in the zinc concentration of the adrenals following administration of cortisone or ACTH to both normal and hypophysectomized rats. These authors postulated a possible relationship between the activity and zinc concentration of these glands. Whether the increased zinc concentration is associated with hyperactivity of the adrenal is not clear from the data they presented. Very recently Prasad et al. (17) failed to find histochemical enzymatic changes in the adrenals of zinc-deficient rats.

The need for determining the degree of infestation and infection in commercially available squirrel monkeys is apparent from this study. Furry (24) found a high incidence of helminth infestation in 45 "normal" squirrel monkeys and our experience with our animals was similar. In the present study, the monkeys fed the low zinc diet grew poorly in comparison with the controls although both groups were parasitized to the same degree. The individual data do suggest that parasitic infection may have aggravated the weight loss of some animals fed the zinc-deficient diet, and may have resulted in suboptimal growth in some of those fed the zinc-supplemented diet.

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Parakeratosis of the Tongue — a Unique Histopathologic Lesion in the Zinc-Deficient Squirrel Monkey [1]

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ABSTRACT A study was made of the pathology in zinc-deficient monkeys. Lesions of the mucosal lining in the tongues of 9 male, weanling, squirrel monkeys (*Saimiri sciureus*) fed a low zinc diet were identified in 2 experiments. They were compared with controls given the same diet supplemented with 15 μg of zinc/g of diet. Tissue changes of the epidermal mucosa of the monkeys fed the unsupplemented diet were characterized by hyperplasia which was more extensive over the anterior dorsum and less prominent on the ventral surface. Parakeratosis was present particularly over the anterior dorsum of this organ with keratohyalin granules absent or comparatively sparse in many regions. Atrophy of the underlying muscle fibers was found in one monkey fed the low zinc diet.

The pathology of zinc deficiency has been described in many species (1–5), but the changes occurring in the experimental primary deficiency of this metal have not been described in primates. Studies in Egypt and Iran recently describe a syndrome characterized by dwarfism and hypogonadism thought to be associated with zinc deficiency in human males (6, 7). Since schistosomiasis, hepatosplenomegaly, iron deficiency and other diseases complicated the picture in these subjects, it is important to attempt to identify lesions peculiar to zinc deficiency which might prove valuable in assessing zinc nutrition in man. In this respect it seemed worthwhile to determine the effects of a primary zinc deficiency in primates.

METHODS

A more detailed account of the methods is given in another report (8). Twenty-two male, weanling squirrel monkeys (*Saimiri sciureus*), weighing approximately 300 g or less, were purchased from commercial sources.[2] Eleven of these were fed a low zinc diet containing in grams per 100 g: zinc-low casein, 18 (8); cottonseed oil,[3] 10; mineral mix,[4] 3.82; vitamin mix,[5] 2; choline chloride, 0.2; ascorbic acid, 0.1; and sucrose 65.88.[6] A second group of 11 monkeys were fed the same diet supplemented with $ZnSO_4 \cdot 7H_2O$ to provide an additional 15 μg of zinc/g of diet. In the first trial, 6 monkeys, 3/group were studied for 330 days. In the second trial, 16 monkeys were fed the diets for 60 to 130 days. They were housed in all-Plexiglas [7] cages made locally.[8] Diet and deionized water were provided in plastic cups. The monkeys were housed individually in temperature-controlled rooms at 27° and 45% humidity.

The monkeys fed the zinc-supplemented diet showed progressive increases in body weight. Those animals given the low zinc diet, in general, slowly lost weight. Estimates of the daily food intake of each monkey were made by weighing the diet not consumed. The intakes so recorded

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[2] Tarpon Zoo, Tarpon Springs, Florida and the Pet Farm, Miami, Florida.

[3] Vitamin A, 3250 IU (Aquasol A, U. S. Vitamin and Pharmaceutical Corporation, New York), Vitamin D_2, 650 IU (Drisdol, Winthrop Laboratories, New York), and γ-tocopherol, 20 mg (Nutritional Biochemicals Corporation, Cleveland), were added to cottonseed oil (Wesson Oil, Hunt-Wesson Foods, Fullerton, California).

[4] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 J. Nutr., *14*: 273. The following salts were also added: (in μg) $CoCl_2 \cdot 6H_2O$, 32; $Na_2SeO_3 \cdot 5H_2O$, 27; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 7.4.

[5] Mann, G. V., personal communication, Vanderbilt University, Nashville, Tennessee. Each 2 g contained: (in mg) thiamine·HCl, 1.5; riboflavin, 1.5; pyridoxine·HCl, 1.5; Ca pantothenate, 9.0; biotin, 0.02; folic acid, 0.5; vitamin B_{12}, 0.0025; niacinamide, 22.5; inositol, 20; menadione, 10; and sucrose to make 2 g.

[6] Godchaux Sugar Refining Company, New Orleans, Louisiana.

[7] Owens-Corning Fiberglas, Toledo, Ohio.

[8] Economy Plastics, Nashville, Tennessee.

were on the high side because it was not possible to recover all of the uneaten diet. Since anorexia was not apparent, except terminally, we consider that inanition did not contribute to the described pathology.

Tissues were taken for histologic studies after the monkeys were killed with sodium pentobarbital. These tissues were fixed in 10% neutral formalin, dehydrated in a graded series of alcohols, cleared in xylol, and infiltrated and embedded in Paraplast.[9] They were sectioned at 6 μ and stained with either the hematoxylin and eosin, periodic acid-Schiff, or the Mallory aniline blue collagen procedures (9). The tissues examined by light microscopy, included lung, liver, kidney, spleen, pancreas, adrenal, pituitary, tongue, esophagus, stomach, small intestine, testes, epididymes, anorectal junction, skin of the knees and lateral sides of the thigh.

RESULTS

Pathologic changes were consistently observed in the tongues of the monkeys fed the low zinc diet. All 8 monkeys in the second experiment showed distinct thickening of the mucosal lining of this organ which was particularly prominent over the anterior dorsum and less marked on the ventral surface. The cells of the mucosa were increased in number. Those in the basal layers were crowded and their nuclei were hyperchromatic. They formed large pegs that extended deep into the submucosa and had a clubbed appearance in certain regions. Parakeratosis was more evident over the anterior dorsum of the tongue. In the first experiment the tongue of one monkey fed the low zinc diet for 330 days was examined and showed thickening of the mucosal lining of this organ. In the tongues of the zinc-supplemented groups, keratohyalin granules were numerous, particularly in the cell layers subjacent to the filiform papillae. In the tongues of 8 monkeys in the second experiment fed the unsupplemented diet, these granules were comparatively sparse and were absent in many regions. These differences are illustrated in figures 1 and 2. The underlying muscle fibers, particularly in one monkey representing the deficient group were atrophic (fig. 3).

In the lungs of the monkeys, pathologic changes were found on occasion. These consisted of sparse infiltrations of larvae with interstitial proliferations which were similar in morphology to those of strongyloides. Both the control and deficient groups of monkeys were affected similarly. Some of the animals whose spleens appeared enlarged showed prominence of white pulp, suggesting hyperplasia of the lymphoid nodules. The enlarged livers of some monkeys showed no cellular changes, except for one monkey fed the low zinc diet in which fatty metamorphosis was seen in the liver and the kidneys. The testes in both the deficient and control groups showed immature seminiferous tubules. In 5 monkeys, the testes were still undescended at autopsy. The histology of other tissues examined, including those of the esophagus and skin, were comparable in both experimental groups.

DISCUSSION

In a summary of the pathologic changes seen in the tongue in different nutritional diseases, Follis (10) indicates that a common histological finding is papillary atrophy, and because it occurs in various states such changes are considered nonspecific. In contrast with the atrophic changes observed in other deficiencies of man, the histological lesion in the mucosa of the tongue in the present study consists of marked thickening and hyperplasia. It would be of interest, in view of these findings, to examine mucosal biopsies of the tongue of humans who might be affected with zinc deficiency.

Similar cellular changes were seen in the posterior part of the tongue, posterior roof of the mouth, esophagus, forestomach and skin of zinc-deficient rats, suggesting that the lesions in these monkeys are related specifically to zinc deficiency. Although there is no formal description of parakeratosis of the tongue in other species, comments in the literature indicate that it may occur in zinc-deficient pigs (11).[10] In primates, some of the epithelial cells of the tongue, particularly those capping the filiform papillae, are fully kerati-

[9] Fisher Scientific Company, New York

[10] Luecke, R. W., personal communication, Michigan State University, East Lansing, Michigan.

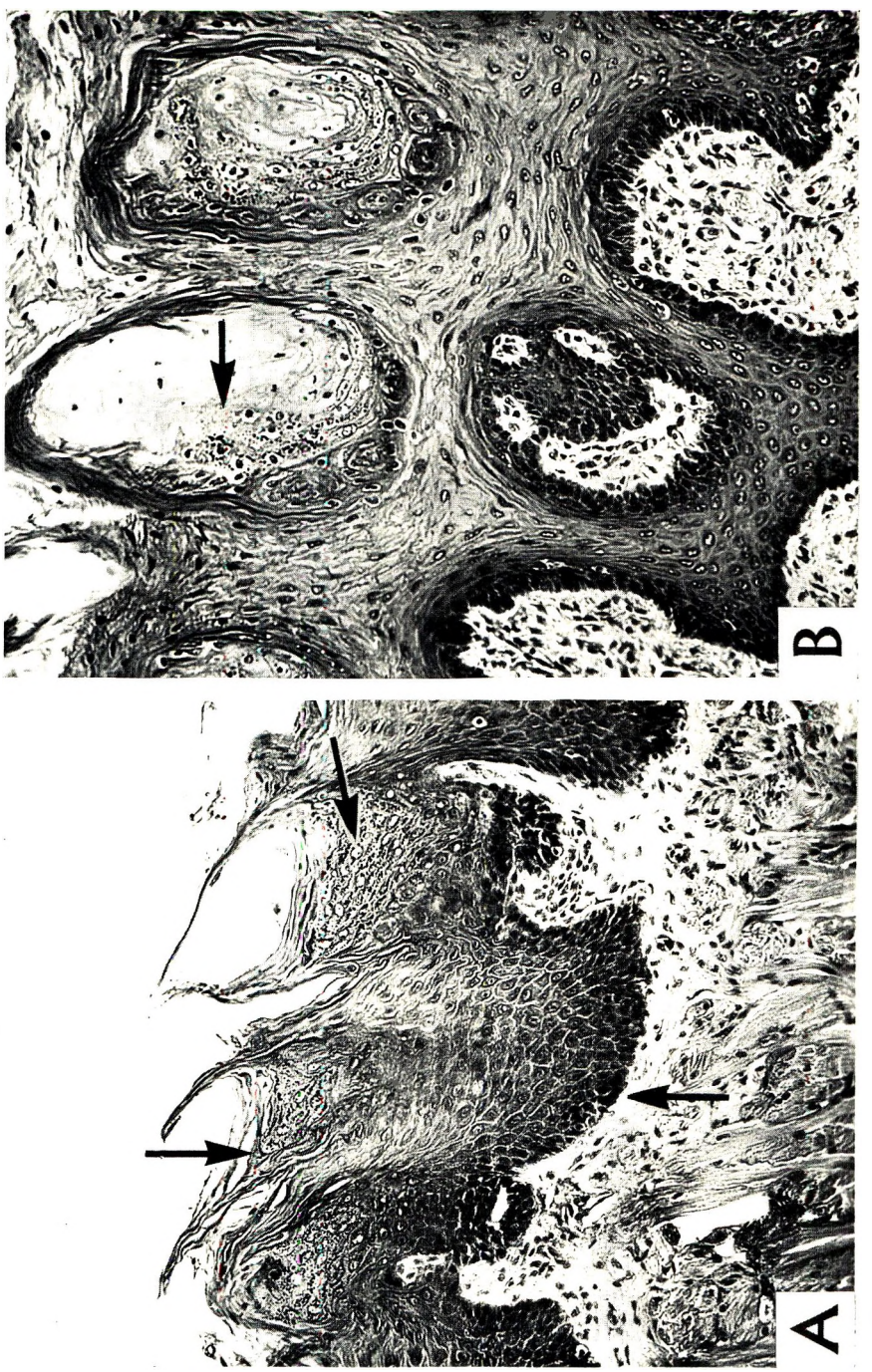

Fig. 1 *A*. Photomicrograph of section of anterior dorsum of the tongue of a control monkey. Note thickness of mucosal layer between two left arrows. Upper arrows point to numerous keratohyalin granules at base of papillae. The latter are capped by keratinized cells. × 100. *B*. Photomicrograph of section of anterior dorsum of the tongue of a monkey fed a low zinc diet. There is clubbing of the rete pegs and keratohyalin granules are less (arrow) than those seen in the mucosa of the control. × 100.

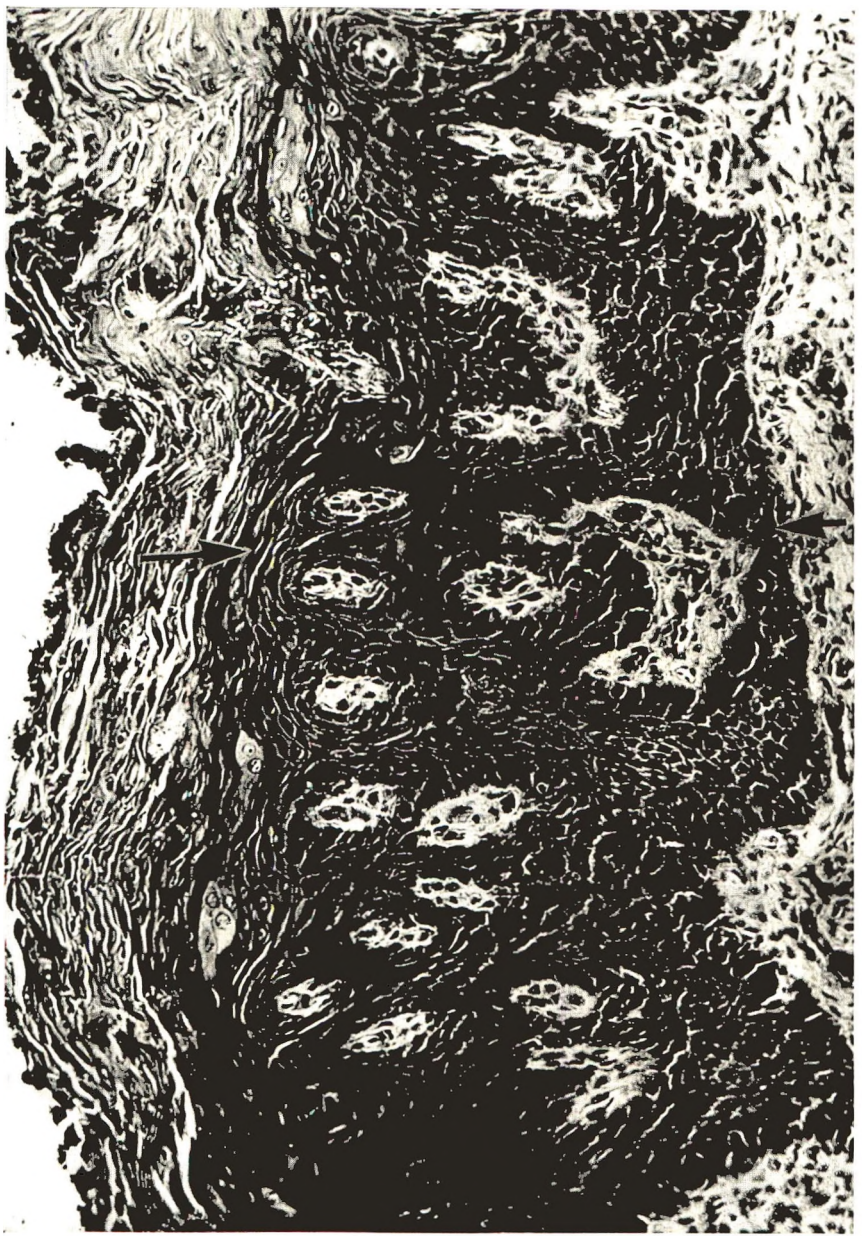

Fig. 2 Photomicrograph of a section of anterior dorsum of the tongue of a monkey fed a low zinc diet. The mucosal lining between the arrows is greatly thickened. There is parakeratosis above upper arrow. Papillae are not discernible and no keratohyalin granules are seen. × 100.

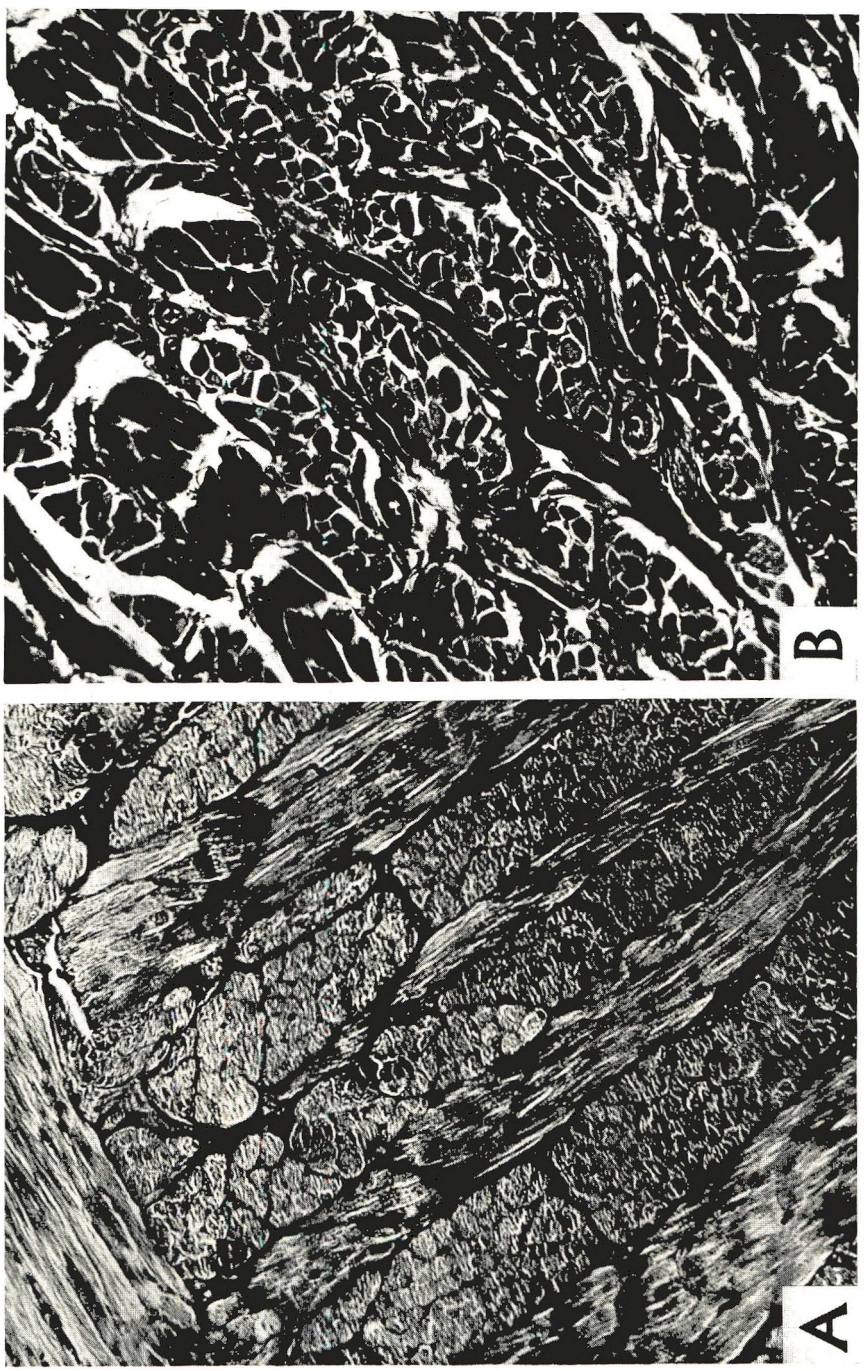

Fig. 3 *A*. Photomicrograph of a section of the tongue of a monkey fed a zinc-supplemented diet illustrating normal appearance of muscle fibers. × 100. *B*. Photomicrograph of section of the tongue of a monkey fed a low zinc diet illustrating atrophy of muscle fibers. × 100.

nized (12), while the mucosa of the esophagus does not undergo complete keratinization (13). The esophagus of the rat is lined by a horny, keratinized layer, and it is probable that the failure to demonstrate a parakeratotic change in the esophagus of the monkey may be related to species difference in the histology of the alimentary tract.

The sparsity of keratohyalin granules in the tongues of the zinc-deficient monkeys deserve comment. Ultrastructural studies by Brody (14, 15) indicate that the tonofibrils in the human and guinea pig epidermis are oriented around these granules, suggesting that either one might be a precursor state in the development of the keratin pattern. Spearman (16), on the other hand, considers keratohyalin granules to be the product of cytolysis. Nevertheless, this author suggests that since these granules have the capacity to adsorb enzymes and minerals, they might influence keratinization. Interestingly, exogenous zinc under certain in vitro conditions, binds with marked affinity to the region of the keratohyalin granules (17, 18). The findings in the present study suggest that experimental zinc deficiency in animals might prove useful as a tool in defining further the relation of these granules to keratinization.

Furry (19), in a study of histopathology and parasitology of 45 squirrel monkeys, found helminthic infections and various inflammatory lesions. Accordingly, this author emphasized the need to consider the presence of such infections in evaluating the results of experiments using this primate. In the present study, both the control and zinc-deficient groups appear to be parasitized to the same degree. Although the monkeys fed the low zinc diet lost some weight in comparison to the controls, the weight differences were relatively small. Overt inanition in the zinc-deficient group was not observed except terminally. We consider the lesion of the tongue to be characteristic of zinc deficiency in the primate unrelated to inanition or to parasitism.

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Yellow Pigments Excreted by Vitamin A-depleted Sheep [1]

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ABSTRACT The possibility of gastrointestinal synthesis of vitamin A precursors which would benefit sheep was investigated. Wethers that had been fed a low carotene ration for 30 months were found to excrete from 1.6 to 3 times their carotene consumption. Results of spectrographic analysis of the excreted pigment indicated that it was not pure β-carotene. The pigment was neither beneficial nor toxic when fed to vitamin A-depleted chicks at levels equivalent to NRC requirements for β-carotene. Possible absorption of carotene from the cecum or colon was studied by injecting ^{14}C-labeled β-carotene into ligated loops of the cecum and colon of four anesthetized wethers. Although average recovery of radioactivity from ether extracts of the loops was only 86%, activity was not detected in the ether extracts of venous blood, liver, or kidneys and it was concluded that little carotene was absorbed under these conditions.

Moore (1) states that he has not observed a practical case of vitamin A deficiency in sheep. At the beginning of the work reported here, wethers had been maintained since weaning for a period of 30 months with a diet low in carotene and vitamin A. The wethers had not shown gross deficiency symptoms during this time and a full explanation for their survival on this low intake was not readily available. The requirements for vitamin A tend to be directly related to body weight, but many factors influence the efficiency of utilization and the requirement for vitamin A precursors. It would be desirable to know why sheep can exist with levels of vitamin A that might be disastrously inadequate for other ruminants.

Sheep (2) and goats (3) have been reported to excrete more carotene than they consumed, and some evidence exists for intestinal microbial synthesis of carotene. Thus, microbial synthesis of carotene in the large intestine might partially explain the survival of sheep with low carotene intakes for prolonged periods. *Phycomyces blakesleeanus* (4) *Blakeslea trispora* (5) and various purple bacteria (6) synthesize carotenoids in vitro. The use of labeled acetate (7) and labeled leucine (8) substrates have been shown to result in radioactive carotene. However, microbial synthesis of carotene has not been clearly demonstrated in vivo. The present experiments were designed: 1) to determine the carotene balance of sheep maintained with a low carotene intake, 2) to ascertain the biological potency of the yellow pigments excreted by sheep, and 3) to measure carotene absorption from the large intestine as a help in evaluating the benefit to sheep of possible microbial synthesis of vitamin A precursors.

EXPERIMENTAL METHODS

Carotene balance. A carotene balance study was conducted using six vitamin A-depleted wethers that had been maintained at this station for more than 2 years with a ration of wheat straw and soybean meal. Liver biopsy obtained by the method of Dick (9) confirmed severe vitamin A depletion in these wethers (table 1), but the vitamin A levels in the serum (19.5 to 32.5 μg/100 ml) were not greatly different from those of animals receiving normal rations. The liver samples, 2 to 3 g wet weight, were digested in 10% alcoholic potassium hydroxide with subsequent extraction with petroleum ether. Vitamin A was determined by the trifluoroacetic acid color reaction (10). Serum levels of vitamin A were determined from jugular blood extracted by the method of Kimble (11) and color was developed as above.

The experimental ration was a mixture of 57% corn cobs, 37% milo, 6% soybean meal and minerals calculated to provide

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TABLE 1

Vitamin A status of wethers used in the carotene balance study

Wether no.	Initial [1] wt	Initial [1] serum vitamin A	Final [2] serum vitamin A	Initial [1] liver vitamin A
	kg	*μg/100 ml*	*μg/100 ml*	*μg/g*
1	40.9	27.8	17.7	—
2	41.8	22.2	15.6	1.12
3	40.9	27.8	27.8	1.73
4	31.8	30.2	15.6	1.32
5	44.5	19.5	17.7	1.85
6	43.6	32.3	19.5	1.84
Mean	40.5	26.6	19.0	1.57
SE		1.9	1.8	0.14

[1] Status after fed a ration of wheat straw and soybean meal for 2 years.
[2] Status after conducting the carotene balance.

the known requirements (12) except for vitamin A precursors. The animals were adapted to the ration for 2 weeks and placed in metabolism crates for 10 days of fecal collections. Daily aliquots of the feces were frozen for later analysis. The intake of carotenes by these wethers was determined by extraction of feed with a 70:30 hexane-acetone mixture for 24 hours at 22° followed by chromatography through commercially prepared magnesia columns as described by standard AOAC (13) procedures for carotenes. The excretion of carotenes was determined by extraction of the feces with 70:30 hexane-acetone as in the feed analysis, but additional purification of the crude fecal extract was necessary before chromatography. The water-soluble phase was removed by washing with equal volumes of water in a separatory funnel. The fat-soluble phase was subjected to saponification in a 10% aqueous potassium hydroxide suspension at 22° with agitation for one hour. The residue was removed by centrifugation and the supernate containing nonsaponifiable fat-soluble pigments was reduced to 65 ml in vacuo. The last trace of water was removed by anhydrous ammonium sulfate; then the preparation was chromatographed through magnesia columns and the eluent read at 436 mμ for comparison with a β-carotene standard.

Spectrographic evaluation. Spectrographic analysis of the fecal pigments was made with a recording spectrophotometer. The amount of ubiquinone in the fecal extract was estimated on the basis of its absorbency at 275 mμ when reduced with borohydride (14).

Bioassay. The vitamin A potency of the fecal pigments was assayed by feeding the pigment to vitamin A-depleted chicks. The pigments from feces collected during the balance trial were extracted and concentrated in vacuo for purification by saponification and countercurrent distribution. Final preparation for feeding was accomplished by removing the solvent with a rotary evaporator in vacuo and redissolving in cottonseed oil. To establish a vitamin A growth response, 4 groups of chicks, 8/group, were fed a chick basal ration plus vitamin A palmitate at levels of 12.5, 25, 50, and 100% of the NRC (15) requirement for chicks. Three other groups of depleted chicks were fed the basal ration plus the fecal pigments at theoretical levels (based on the optical density of β-carotene) of zero, 50, and 100% of the requirement. To test the pigment for possible growth depression or toxicity to chicks, it was fed with adequate vitamin A at twice the highest level used in the assay. Growth rates were compared with those of the control group.

Absorption from cecum and colon. An additional experiment was conducted to study the absorption of ^{14}C-labeled β-carotene from the cecum and colon of sheep. Four mature wethers, considered to have normal nutritional status, were anesthetized and sections of the cecum and colon were exposed, ligated and injected with 66, 165, or 330 μg of ^{14}C-labeled β-carotene containing 0.86, 1.27 or 2.54 μCi in a suspension of polyoxyethylene sorbitan monooleate (Tween 80). Jugular blood, taken at zero, 30, 60, 90, and 120 minutes, was alcohol-precipitated, ether-extracted and prepared for liquid scintillation counting. At the termination of the absorption period the liver, kidneys, and the ligated loops with contents were excised. Determinations of radioactivity in the liver and kidney samples (2–3 g wet weight) and the entire ligated loops with contents were made from ether-extracted alcoholic potassium hydroxide digests.

RESULTS AND DISCUSSION

The initial vitamin A-depleted status of these wethers and the low carotene diet

TABLE 2

Carotene balance of depleted wethers fed low carotene ration

Sheep no.	Total carotene intake	Total carotene excreted	Balance
	μg/day	*μg/day*	*μg/day*
1	202	598	−396
2	202	393	−191
3	202	674	−472
4	202	436	−234
5	202	356	−154
6	202	334	−132
Mean	202	465	−263
SE			56

should have allowed detection of small quantities of carotene that might be synthesized by intestinal microorganisms. During the balance trial, the wethers excreted from 1.65 to 3.00 times (132 to 472 μg) the quantity of carotene consumed (table 2). This supports the findings of McGillivray (2) and Majumdar and Gupta (3) and strongly suggests microbial synthesis of carotene, but the apparent synthesis of carotene did not maintain serum levels of vitamin A which decreased an average of 28% (table 1). This decrease, occurring after the animals had been fed a diet of wheat straw and soybean meal for 2 years, might reflect the somewhat lower carotene intake with the experimental ration, 0.306 versus 0.202 mg of dietary carotene, respectively. However, these wethers having almost no liver stores might be expected to exhibit downward fluctuations in serum levels of vitamin A due to stresses that would be of little consequence to normal animals. The apparent lack of benefit from the synthesized pigments suggested that the excreted pigment might represent carotenes with low biological potency or that the site of synthesis in the gastrointestinal tract was not favorable for absorption.

An indication of potential vitamin A potency was provided by more detailed spectrographic evaluation of the fecal pigment. The results indicated that it was not pure β-carotene (fig. 1). The absorption between 425 and 500 mμ was similar to a carotene absorption curve, but below 400 mμ the fecal pigment was much more absorbent than carotene dissolved in *n*-hexane. The strong absorbency below 400 mμ suggested the presence of ubiquinone, which has a

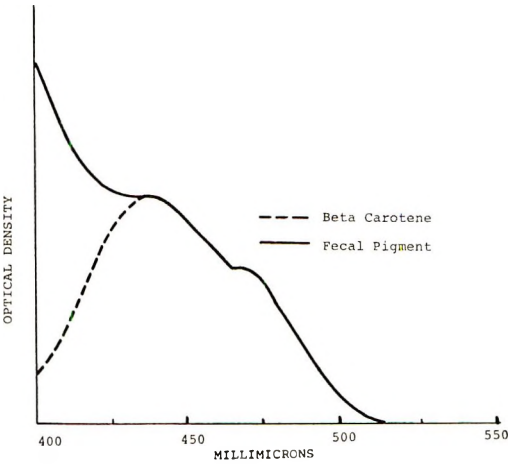

Fig. 1 Absorption spectra of the fecal pigment from carotenoid balance trial.

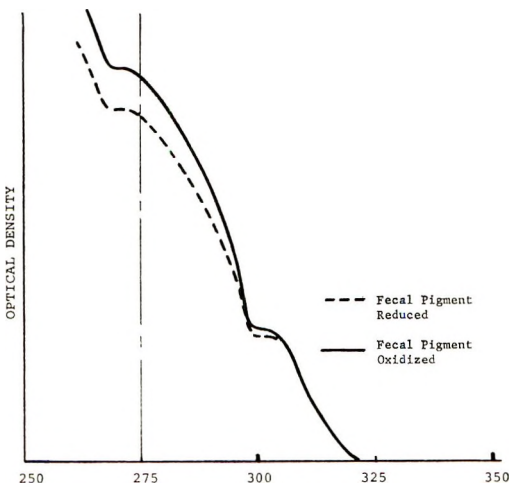

Fig. 2 Absorption spectra of the fecal pigment showing the shift in optical density at 275 mμ when reduced with borohydride.

reported maximum at 275 mμ (14). This fat-soluble isoprenoid compound has been isolated in large quantities from the livers of vitamin A-depleted rats (16). Based on the spectral shift shown in figure 2, ubiquinone was detected but accounted for less than 5% of the total pigment.

A further evaluation of the potential vitamin A potency was obtained by adding the pigment to diets fed to vitamin A-depleted chicks. These chicks responded to vitamin A palmitate with graded levels of growth, but did not respond to the fecal pigment.

Weight losses with high mortality occurred in the chicks receiving the fecal pigment, but results of tests with adequate vitamin A in the diet indicated that the pigment was not growth-depressing at levels twice those used in the assay and other signs of toxicity were not observed. It was concluded that the pigment excreted by the wethers did not possess detectable vitamin A potency.

Lack of vitamin A activity of the excreted pigments does not preclude the possibility that active pigments might have been synthesized and either absorbed or converted to biologically inactive forms before excretion. If the synthesis occurred in the cecum and colon, absorption from these sites would be necessary before the synthesized pigments could be utilized by the sheep. Thus, it was of interest to study possible β-carotene absorption from the cecum and colon.

Recovery of radioactivity in ether extracts of the loops excised after the absorption studies was somewhat variable and averaged 86.2 $\pm$ 7.7% of the recovery from control loops injected with labeled carotene after excision. However, radioactivity was not detected in ether extracts of blood, liver or kidneys of any of the 4 wethers studied. Conversion to forms not extracted by ether might account for some of the apparent loss from the loops. It was calculated that if as little as 0.5% of the carotene injected into the loops were absorbed in the blood, 0.2% in the liver or 0.1% in the kidneys, activity in the counted samples would have been an easily detected 30 to 50 cpm (80% counting efficiency) higher than the background of 20 cpm. Thus, it appears that carotene was not efficiently absorbed from the cecum or colon at any of the 3 levels tested. Although the blood supply to the ligated loops was not interrupted and motility was observed throughout the experiment, the conclusiveness of the absorption study is limited by possible effects of anesthesia and surgery.

The results of these experiments confirm previous observations of negative carotene balance in sheep fed low carotene diets. They provide little support for the theory that this is a result of microbial synthesis in the cecum and colon which might supply a significant portion of the vitamin A requirement. The results might be different for sheep fed other diets or having different microorganisms in the cecum and colon, but failure to obtain convincing evidence of carotene absorption from these sections of the digestive tract does not encourage this speculation.

ACKNOWLEDGMENTS

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Availability to the Chick of Zinc·Phytate Complexes Isolated from Oil Seed Meals by an in vitro Digestion Method [1,2]

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ABSTRACT Studies were made of the form in which zinc was found after digestion in vitro of oil seed meals which varied in zinc availability in vivo. Regardless of in vivo differences, the zinc of two sesame meals and one safflower meal was present in an insoluble, nondialyzable Ca·Mg·Zn·phytate complex at intestinal pH. The zinc was little available to the chick as shown by low uptake of ^{65}Zn from the labeled complexes. For a soybean meal digest, about 75% of the extracted zinc, 40% of the phytate phosphorus and 90% of the calcium and magnesium were soluble at intestinal pH. About 50% of the zinc was dialyzable in a 4-hour period. The zinc of the soybean meal digest was bound in a water-soluble and dialyzable complex which was more stable than zinc phytate. The binding agent was termed a "carrier." The addition of soluble digestion products of the sesame or safflower meals to the insoluble labeled Ca·Mg·^{65}Zn·phytate complexes did not promote dialysis of ^{65}Zn, but the "carrier" rendered 15 to 29% of the ^{65}Zn dialyzable at intestinal pH. Three isolated soy proteins contained low or no "carrier" properties.

Many oil seed meals contain enough zinc so that if it were all available the dietary requirement of the chick would be readily met. Phytic acid, as the calcium·magnesium salt, is also present in the meals. Addition of phytic acid to casein basal diets increased the dietary requirement for growth of the chick or rat (1–3); increasing the dietary calcium also led to a further increase (3–5). Other workers using a smaller amount of phytic acid with animal proteins or protein hydrolysates did not obtain a zinc deficiency in chicks (6) nor a decrease in the absorption of oral ^{65}Zn by the rat (7). When a prepared zinc phytate was fed to poults the availability of the zinc varied (8). In some trials, the zinc phytate produced growth comparable to zinc oxide; in others it was less available.

In this laboratory the amount of zinc available from oil seed meals for growth and prevention of leg deformities in chicks varied widely (9). A relationship between zinc availability and the zinc, calcium, magnesium or phytate content of a meal was not found.

Previous work [3] had shown that the zinc extracted at pH 1 from a strong zinc-binding sesame meal was poorly utilized. Subsequently, the zinc was found to be associated with an insoluble phytate complex. The possibility was considered that differences in the form in which the zinc of the meals was available for absorption from the intestine could explain the in vivo differences. Since binding forces between ions and ligands are less at a lower pH, a zinc phytate complex, present in the oil seed meal, could be dissociated in the chick's proventriculus during digestion. This offered the opportunity for combination of the zinc with constituents or digestion products of the protein-rich meals at intestinal pH. Zinc could be more available in vivo from the new complexes.

The first objective was to determine by in vitro studies the form in which zinc of oil seed meals is present in the chick's digestive tract. Depending on the results, the further problem would be concerned

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[2] Presented in part at the meeting of the Federation of American Socities for Experimental Biology in Chicago, April 1967.

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either with the factors which influence the availability of zinc from a phytate complex during digestion and absorption, or with the availability of zinc from some complex which formed a more stable compound with zinc than did phytate during digestion. While the present report is concerned primarily with the first objective, some exploratory studies of the other objectives are reported.

An in vitro digestion technique, simulating the initial digestive processes of the chick, was used with 4 meals which varied in availability of zinc in vivo. An evaluation of some of the studies in vitro was carried out by feeding trials with chicks.

MATERIALS AND METHODS

Variations in the phytate phosphorus, zinc, magnesium and calcium content of the meals tested were observed (tables 1 and 2). The addition of 60 ppm of zinc to Texas 61 sesame meal, 15 to 30 ppm to safflower meal and 0 ppm to Venezuelan sesame meal rations was necessary to prevent leg deformities and promote growth in chicks (9). When soybean meal rations were fed, leg deformities were not found; growth response to added zinc varied with the meal sample (9).[4] When the studies were initiated a soybean meal was used which gave as good growth without added zinc as when 30 ppm were added. The results reported here were obtained with a soybean meal which gave significantly ($P < 0.05$) better growth when 30 ppm of zinc were added to the ration (10). The zinc content of the 2 meals (54 ± 4 ppm) showed little difference on repeated analyses.

In vitro digestion. The sesame and safflower meals were extracted with cold hexane to a fat content of 1 to 2% and ground to pass through a 40-mesh screen. A water slurry of the meal (10:1 v/w) was adjusted to pH 3, the pH of the chick's proventriculus and gizzard (11), with 6 N HCl. Pepsin (2 g/100 g of meal) was added and digestion carried out for 15 minutes at 40° with constant stirring. The digestion time was based on the passage time of similar oil seed rations from the beak to the upper part of the intestine.[5] After a 10-minute centrifugation at 2500 rpm, aliquots of the supernatant extract (pH 3 extract) were taken for analysis. A 10% solution of Na_2CO_3 was added to the remaining pH 3 extract to bring the pH to 6.8, the pH of the chick's intestine (12). A copious, gelatinous precipitate appeared in the extracts of the sesame and safflower meals. Less precipitate appeared with soybean meal. The precipitates were centrifuged and washed twice with a small quantity of water with intermittent centrifuging. Aliquots of the precipitates and supernatants were taken for analysis.

Dialysis. $^{65}ZnCl_2$ was added to the digestion extract at pH 3. The pH was adjusted to 6.8, and 200 mg of pancreatin plus 100 mg of erepsin were added to each equivalent of 10 g of meal. The slurry, 70 to 80 ml, was placed in cellophane tubing and dialyzed against 200 ml of water at 40° in a water-bath shaker for 4 hours. The activity of 2 ml of the total pH 6.8 slurry, of its precipitate and supernatant, and of 2 ml of the dialysate was measured in a Baird Atomic Model 709 well scintillation counter.

Proportions of about one to three between the bag contents and the dialyzing medium would permit about 25% retention and 75% passage of the zinc on dialysis. When dialyzing time was increased to 16 hours, those samples from which zinc dialyzed approached this maximum. Samples from which little zinc dialyzed after 4 hours did not show an appreciable increase in dialyzed zinc at 16 hours. Therefore, 4 hours were used as the dialyzing time.

In some trials, an equivalent amount of the pH 6.8 supernatant of soybean meal was mixed with the labeled pH 6.8 digest

[4] The meals supplied 20% protein; the fat content was adjusted with corn oil to total 10%. The sesame meals were supplemented with 0.9% L-lysine·HCl, the safflower meal with 0.75% L-lysine·HCl and 0.25% MHA·Ca (methionine hydroxy analogue calcium 90%, Dupont, Wilmington, Delaware) and the soybean meal with 0.33% MHA·Ca. All rations were supplemented with 0.3% choline·Cl (70%), a vitamin mix which supplied/kg of ration: (in mg) menadione bisulfite sodium, 4.5; biotin, 0.2; pyridoxine·HCl, 5; folic acid, 5; riboflavin, 10; Ca D-pantothenate, 30; thiamine (mononitrate), 10; niacin, 50; vitamin B_{12}, 0.02; and 1500 ICU vitamin D_3; 10,000 IU vitamin A (vitamin A palmitate); 50 IU vitamin E (*d*-α-tocopheryl acetate). The mineral mix supplied/kg of each ration: (in g) $CaHPO_4 \cdot 2H_2O$, 27.2; $CaCO_3$, 14.0; K_2HPO_4, 11.1; NaCl, 6.0; and (in mg) $MgCO_3$, 175; Fe citrate, 333; $MnSO_4 \cdot H_2O$, 333; KI, 2.6; $CuSO_4 \cdot 5H_2O$, 16.7.

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TABLE 1

Zinc and phytate P content of successive in vitro digestion fractions of oil seed meals (each equivalent to 1 g of original meal) [1]

	Sesame				Safflower		Soybean	
	Texas 61		Venezuelan					
	Zn [2]	Phytate P [3]	Zn [2]	Phytate P [3]	Zn [2]	Phytate P [3]	Zn [2]	Phytate P [3]
	μg	*mg*	*μg*	*mg*	*μg*	*mg*	*μg*	*mg*
Original meal	155 ± 5	10.0 ± 1	136 ± 0	9.9 ± 0.5	153 ± 3	10.7 ± 1	58 ± 2	4.2 ± 0.4
pH 3 extract	66 ± 4	2.42	50 ± 0	2.88	80 ± 1	4.74	30 ± 4	0.95 ± 0.05 [4]
pH 6.8 precipitate	66 ± 1	2.42 ± 0.05 [4]	50 ± 0	2.88 ± 0.09 [4]	84 ± 1	4.74 ± 0.15 [4]	7 ± 0.5	0.60 ± 0.02 [4]
pH 6.8 supernatant	tr	0	tr	0	tr	0.14 ± 0.02 [4]	20 ± 1	0.35 ± 0.02 [4]

[1] The 4 meals were treated as a set for digestion and analysis. Aliquots equivalent to 10 g of the hexane extracted original meal were taken of each fraction.
[2] Duplicate aliquots of the digestion fractions were dried at 50°, ashed at 600°, dissolved in 0.1 N HCl and the zinc was measured by atomic absorption spectrophotometry.
[3] The phytate phosphorus, in duplicate on duplicate aliquots of the digestion products, was measured by modifications of the method of Pringle and Moran (12). The value of the pH 3 extract of the sesame or safflower meals was calculated from those of the pH 6.8 precipitate plus the supernatant. The soybean meal fractions were so diluted that the amount of phytate P measured was comparable to that of the other meals.
[4] Mean ± SE.

TABLE 2

Division of Zn, Ca and Mg between successive digestion fractions of oil seed meals, each equivalent to 1 g of the original meal (by analysis) [1,2]

	61 Sesame			Venezuelan sesame			Safflower			Soybean		
	Zn	Ca	Mg	Zn	Ca	Mg	Zn	Ca	Mg	Zn	Ca	Mg
	μg	*mg*	*mg*	*μg*	*mg*	*mg*	*μg*	*mg*	*mg*	*μg*	*mg*	*mg*
Original meal	155 ± 5 [3]	1.7 ± 0	8.8 ± 0.1	136 ± 0	11.3 ± 0	8.6 ± 0.6	150 ± 3	4.0 ± 0.1	7.9 ± 0	58 ± 2	2.1 ± 0.2	2.3 ± 0.1
pH 3 extract [4]	76 ± 1	1.2 ± 0.2	5.2 ± 0.3	64 ± 1	2.6 ± 0.4	4.9 ± 0.2	99 ± 2	1.3 ± 0.1	5.7 ± 0.2	33 ± 3	1.5 ± 0.1	2.2 ± 0
pH 6.8 precipitate [4]	76 ± 1	1.1 ± 0.3	2.1 ± 0.2	65 ± 3	1.8 ± 0.3	1.8 ± 0.2	105 ± 10	1.3 ± 0.2	3.1 ± 0.3	7 ± 0.05	0.24 ± 0.02	0.26 ± 0.02
pH 6.8 supernatant [5]	tr [6]	0.2 ± 0	3.1 ± 0.2	tr [6]	0.7 ± 0	3.2 ± 0.4	tr [6]	0.1 ± 0	2.4 ± 0.2	27 ± 1	1.32 ± 0.16	1.9 ± 0

[1] Digestion aliquots equivalent to 10 g of hexane extracted meal were not dried but adjusted to 0.1 N HCl and analyzed by atomic absorption spectrophotometry. $SrCl_2$ was added to the aliquots taken for Ca analysis, equivalent to 0.3% Sr, to minimize interference by phosphorus.
[2] Analysis for copper of the meals or fractions by atomic absorption spectrophotometry indicated that only traces were present.
[3] Average and range of 2 analyses.
[4] The high values for Zn and Mg in comparison with those of tables 1 and 3 are ascribed to better extraction due to the use of a mechanical stirring device during digestion. 3.5 ± 0.1 mg of phytate P were extracted from 61 sesame by this procedure; 3.2 ± 0 mg from Venezuelan sesame and 0.9 ± 0 mg from soybean meal. Sufficient safflower meal was not available to obtain a value.
[5] The pH 6.8 supernatants of the sesame meals contained no phytate P; the safflower supernatant contained a trace.
[6] When the digests were labeled with ^{65}Zn for the dialysis trials, 1% or less of the activity appeared in the supernatant.

or pH 6.8 precipitate of the protein sources and dialysis of ^{65}Zn measured. Three isolated soy proteins [6,7,8] were digested and the effect of their pH 6.8 supernatants similarly tested.

As a variation in the general procedure, copper as a 1% solution of $CuSO_4 \cdot 5H_2O$ was added to the labeled pH 3 extract of soybean meal so that copper was present in twice the concentration of the zinc of the original meal. The pH was adjusted to 6.8 and the division and dialysis of ^{65}Zn was measured.

Casein,[9] fish meal [10] and egg white [11] were digested as above, ^{65}Zn added at pH 3 and the subsequent partition and dialysis of ^{65}Zn was measured.

Chick feeding tests. For the ^{65}Zn tracer trials, food was withheld from 2-week broiler type cockerels for 16 hours and the samples force-fed. After 4, 8 and 24 hours, 1 ml of blood (by heart puncture), the liver and left tibia were removed from each of 3 chicks and the ^{65}Zn activity was determined. It has been found in other experiments with ^{65}Zn that maximal uptake in blood usually occurs at 4 hours and in liver from 4 to 8 hours. The ^{65}Zn content of the tibia increases after the initial uptake at 4 hours. By following the ^{65}Zn uptake of the 3 tissues for the 3 time-periods, absorption, liver storage, utilization, as exemplified by bone storage, and excretion could be followed.

RESULTS AND DISCUSSION

Division of zinc and phytate. In the first phase of the in vitro digestion, comparable to that of the chick's proventriculus, (pH 3 extract, table 1), about 40 to 50% of the zinc of the meals was extracted. About one-fourth of the phytate-phosphorus was extracted from the Texas 61 sesame and soybean meals, about one-third from the Venezuelan sesame and one-half from the safflower meal. The difference in zinc availability, in vivo, of the 2 sesame meals was not primarily due to a smaller amount of zinc freed by pepsin digestion nor to a greater amount of phytate extracted from the 61 sesame.

When the pH was adjusted to 6.8, the intestinal pH, the zinc and phytate extracted from the 2 sesame meals were precipitated (table 1). The zinc extracted from the safflower meal and all but a trace of the phytate also appeared in an insoluble precipitate at pH 6.8. A difference in the form in which the zinc of the Venezuelan meal appeared at intestinal pH did not account for its in vivo availability.

With the soybean meal, however, only about one-fourth of the extracted zinc and two-thirds of the phytate phosphorus were found in the pH 6.8 precipitate. About two-thirds of the zinc and one-third of the phytate were soluble at intestinal pH.

Influence of calcium and magnesium content. In vitro studies have shown that uptake of zinc by phytic acid is increased by the addition of calcium and that the resultant complex is less soluble than zinc phytate at intestinal pH (5, 13). The extraction and division of calcium were measured to determine the influence of calcium content on the solubility of the zinc of the meal extracts. Since the chemical behavior of magnesium is similar to that of calcium and the magnesium content of the sesame and safflower meals was high, magnesium distribution was also determined (table 2).

Comparable amounts of calcium were extracted from the 61 sesame, safflower and soybean meals by the pH 3-pepsin digestion. The extract of Venezuelan meal contained a larger amount, reflecting the higher calcium content of the original meal. When the pH was adjusted to 6.8, 70 to 100% of the calcium extracted from the sesame and safflower meals appeared in the insoluble phytate precipitate. With the soybean meal extract, however, only about 16% of the calcium was found in the precipitate. About 90% was soluble along with zinc and phytate.

The magnesium extracted from the sesame and safflower meals was divided between the precipitate and supernatant (table 2). About 90% of the magnesium of the soybean meal was soluble in the supernatant.

The zinc of the sesame and safflower meals was present as a component of or as-

[6] Soybean Protein, Nutritional Biochemicals Corporation, Cleveland.
[7] Assay Protein C-1, Skidmore Enterprises, Cincinnati.
[8] Assay Protein C-1, Archer-Daniels-Midland, Minneapolis.
[9] High Nitrogen Casein, Nutritional Biochemicals Corporation, Cleveland.
[10] Peruvian fish meal, 67.5% protein, 3.09% phosphorus.
[11] Egg White Solids, Armour Creameries, Chicago.

sociated with an insoluble calcium·magnesium·phytate complex. If 6 atoms of zinc were bound to a phytate molecule, about twice as much zinc by weight as phosphorus would be present (392:186). The low amount of zinc in proportion to the phytate phosphorus content of the precipitates might be due to a small amount $Zn_6C_6H_6P_6O_{24}$ or to a phytate molecule which contained both zinc and calcium with or without magnesium or other minerals. The pH 6.8 precipitate of the sesame or safflower meals is referred to as an insoluble calcium·magnesium·zinc·phytate complex in the remainder of this report.

As much calcium was present in the pH 3 extract of the soybean meal as the other meals but an insoluble calcium·zinc·phytate complex was not formed at pH 6.8. The amount of magnesium extracted at pH 3 and thus available for precipitation was comparable to that found in the pH 6.8 precipitate of the 61 sesame meal, but an insoluble magnesium·calcium·zinc phytate was not formed at pH 6.8. It appeared that some substance(s) was present in soybean meal which prevented the precipitation of a large part of the zinc, calcium and magnesium as a phytate complex at intestinal pH.

In vivo availability of the zinc of the calcium·magnesium·zinc·phytate complexes. The in vivo availability of enough zinc from soybean meal to prevent leg deformities might be ascribed to the solubility of a large part of the extracted zinc at intestinal pH, and the unavailability of the zinc of the Texas 61 sesame meal to the presence of an insoluble calcium·magnesium·zinc·phytate complex. An explanation for the in vivo availability of the zinc of the Venezuelan sesame meal and of a small amount from safflower meal did not fit into either class. The in vivo variation could be due to differences in the availability of zinc from the phytate complex or to the effect of other constituents of the meals during further in vivo digestion. To differentiate between these possibilities, the pH 6.8 precipitates of the sesame and safflower meals were labeled with ^{65}Zn (table 3) and fed to chicks. The availability of the zinc of the soybean meal supernatant was not similarly measured due to its large bulk.

A highly significant decrease in the uptake of ^{65}Zn from any of the 3 precipitates by blood, liver or tibia after 4, 8 or 24 hours was found (table 4). A significant difference between the uptake of any of the tissues at any time was not found among the 3 precipitates. As a component of a calcium·magnesium·phytate complex, little zinc was available from any of the precipitates.

Differences in in vivo availability of the zinc of the 3 meals apparently was due to the presence or absence of other components of the meal. For Venezuelan sesame, the components would form a more stable complex with zinc than phytate; safflower meal would contain a lesser amount of these components. They would be absent or bound in 61 sesame meal. The components could either be present after peptic digestion or be freed or formed during

TABLE 3

Constituents of the pH 6.8 precipitates from the equivalent of 10 g of meal

	Texas 61 sesame [1]	Venezuelan sesame [1]	Safflower [1]	Soybean
Air-dried wt, mg	240.0 ± 2	253.7 ± 2	345.3 ± 0.7	180.0 ± 30
Ash, %	47.3 ± 0.5	50.6 ± 1.0	55.7 ± 0.1	19.4 ± 0.2
Zinc,[2] mg	0.66 ± 0.01	0.50 ± 0	0.84 ± 0.01	0.07 ± 0
Magnesium,[2] mg	14.4 ± 0	12.8 ± 0.1	22.7 ± 0	2.2 ± 0.3
Copper [2]	tr	tr	tr	tr
Phytate P, mg [3]	24.23 ± 0.47	28.80 ± 0.9	47.4 ± 1.46	6.02 ± 0.16
Phytic acid equivalent,[4] mg	86.02	102.24	168.27	21.37
Nitrogen [5]	0	0	0	—

[1] Labeled with ^{65}Zn and fed to chicks. Because the precipitates were labeled, the values given are those obtained with the pH 6.8 precipitates of table 2. The same procedure was used.
[2] By atomic absorption spectrophotometry; average and range of 2 analyses.
[3] Average ± SE.
[4] Calculated using 3.55 as a factor.
[5] When no nitrogen was found by a micro-Kjeldahl method, the precipitates were fused with sodium and tested for elemental nitrogen. The soybean meal precipitate could not be washed free from the nitrogen-containing supernatant without losses.

TABLE 4

Uptake of ^{65}Zn from $^{65}ZnCl_2$ and from the pH 6.8 precipitate of Texas 61, Venezuelan sesame or safflower meal labeled with ^{65}Zn [1]

	Retention of ^{65}Zn given								
	Total blood [2]			Liver [3]			Tibia		
Hours	4	8	24	4	8	24	4	8	24
	%	%	%	%	%	%	%	%	%
^{65}Zn [4]	4.7 **	3.9 **	3.4 **	10.3 **	10.6 **	7.5 **	2.6 **	2.7 **	3.3 **
Texas 61 sesame [5]	0.5	0.4	0.7	0.8	0.6	0.7	0.1	0.1	0.2
Venezuelan sesame [5]	0.8	0.4	0.3	0.7	1.0	0.9	0.1	0.2	0.3
Safflower [5]	0.4	0.4	0.2	0.4	0.9	1.2	0.1	0.2	0.3

[1] $^{65}ZnCl_2$ (2.27 Ci/g Zn) was added to the pH 3 pepsin extract, the pH adjusted to 6.8, the resulting precipitate centrifuged, washed once with water, dried at 50°, and placed in a gelatin capsule. Each chick received the equivalent of 10 g of meal containing 25 μCi of ^{65}Zn.

[2] Calculated from the activity of 1 ml of blood, using 10% of the initial weight of the chick as a factor.

[3] Calculated for the total liver from the ^{65}Zn activity of approximately 2-g samples.

[4] 25 μCi of ^{65}Zn plus 0.8 mg of stable zinc as a solution of $ZnSO_4$/chick. The stable Zn addition was based on the zinc content of the pH 6.8 precipitate of safflower meal; 3 chicks/time-period.

[5] Three chicks/time-period.

[6] Before administration of the isotope, the chicks were fed a corn-soybean meal ration for 14 to 16 days containing: (in %) corn, 50; soybean meal, 34; fish meal, 3; alfalfa, 2; whey, 2; calcium, 1; phosphorus (as $CaCO_3$ and defluorinated rock phosphate), 1; Mn, 0.2; NaCl, 0.5; and (in mg/kg) Ca pantothenate, 5; riboflavin, 9; niacin, 20; choline, 200; and vitamin A, 2750 USP units, and vitamin D_3, 530 ICU.

** Significantly different from any of the precipitates ($P < 0.01$).

further intestinal digestion. Since about three-fourths of the extracted zinc of the soybean meal was present in a water-soluble form further digestion might not be necessary for in vivo utilization.

Effect of soluble digestion products during further in vitro digestion and dialysis. The in vitro dialyzing technique simulated the continuous passage of zinc through a semipermeable membrane analogous to the chick's intestine. Pancreatin and erepsin were added to the total pH 6.8 digests (pH 6.8 precipitate + supernatant) of the 4 meals and the dialysis of ^{65}Zn to water measured (table 5). The presence of the pH 6.8 supernatant did not result in dialysis of the phytate-bound zinc of the sesame or safflower meals, whether or not pancreatin and erepsin were present. The in vitro technique was not measuring the conditions which rendered the zinc of the Venezuelan meal available in vivo. With the soybean meal digest, however, dialysis of zinc occurred whether or not pancreatin and erepsin were added.

Conditions governing the solubility and dialyzability of zinc of soybean meal extracts. Since the zinc of the soybean meal supernatant was soluble and dialyzable in the presence of phytate, calcium and magnesium, the conditions under which this occurred were studied. The study might also offer a clue as to the condition necessary on further digestion to render the phytate-bound zinc of the Venezuelan precipitate available in vivo, but which would not affect the zinc of the 61 sesame meal precipitate.

Although about 40% of the phytate occurred in the soybean supernatant in a water-soluble form, when the zinc-containing dialysates were concentrated, phytate was not found. The zinc might be present as a water-soluble salt or bound in a complex which was more stable than zinc·phytate — a "carrier."

Copper forms a more stable compound with many chelating substances than does zinc (14). If zinc were bound to a "carrier" it might be possible to displace it with copper. The zinc could then be taken up by the phytate present and its dialysis reduced.[12,13]

Copper, at twice the amount of zinc in the original soybean meal, was added to the pH 3 extract labeled with ^{65}Zn. When the pH was adjusted to 6.8, 91±2% of the ^{65}Zn was found in the precipitate in contrast to 23 ± 2% when copper was not

[12] Copper also forms complexes with sodium phytate or phytic acid (15, 16) so that zinc might be displaced from phytate as well as the "carrier" and dialysis be increased. However, while the addition of copper to rations containing 61 sesame meal (see footnote 13) resulted in a decrease in the amount of *added* zinc necessary for good growth and prevention of leg deformities, copper had no effect in freeing the zinc of the sesame itself. In the presence of other components of the phytate complex of an oil seed meal, copper phytate may not readily be formed.

[13] Lease, J. G. 1966 Effect of metal ions on uptake of zinc by sesame meal and isolated soy proteins. Federation Proc., 25: 483 (abstract).

TABLE 5

Dialysis of ^{65}Zn from pH 6.8 digestion products labeled with ^{65}Zn after shaking for 4 hours at 40° [1,2]

	pH 6.8 digest		pH 6.8 precipitate				
		+ pancreatin and erepsin	+ H_2O [3]	+ soybean meal-pH 6.8 supernatant [4]	+ isolated soy protein-pH 6.8 supernatant [5]		
	% in dialysate	*% in dialysate*	*% in dialysate* [6]	*% in dialysate*	*% in dialysate*		
Soybean meal	49 ± 4 [7]	51 ± 2					
61 sesame meal	1.1 ± 0.5	1.3 ± 0.1	< 1 [8]	28 ± 3 [9]	6.5 ± 0.3 [10]	1.7 ± 0.2 [11]	< 1 [12]
Venezuelan sesame meal	1.7 ± 0.7	1.6 ± 1	< 1 [8]	27 ± 3 [9]	5.4 ± 0.5 [10]	1.9 ± 0.6 [11]	1.1 ± 0.3 [12]
Safflower meal	1.7 ± 0.7	< 1	< 1	15 ± 1			
Fish meal	< 1	< 1	< 1	31 ± 2			
Casein	< 1 [13]	4.0 ± 2	no precipitate	41 ± 2 [14]		7.2 ± 0.5 [11,14]	
Egg white	14 ± 1	15 ± 2	no precipitate	66 ± 2 [14]			

[1] Percentage of activity of initial sample.
[2] Each equivalent to 10 g of meal or protein; contained 5 μCi of ^{65}Zn added at pH 3, giving about 30,000 counts/5 minutes/2 ml of digest or precipitate slurry. The results in columns 1, 2, and 3 were obtained as a set.
[3] Slurried with an amount of water equivalent to the supernatant.
[4] Slurried with the equivalent of 10 g of soybean meal supernatant. A control as in column 1 or 3 was included.
[5] Slurried with the equivalent of 10 g of the pH 6.8 supernatant of the various soy proteins. A control as in column 4 was included.
[6] Addition of pancreatin and erepsin did not increase dialysis.
[7] Repeated trials gave a range of 49 ± 5% dialysis with 2 different meal samples and 56 ± 5% with mechanical stirring during digestion.
[8] Addition of 50 mg of L-lysine·HCl L-histidine·HCl or L-arginine·HCl in 2 trials did not increase dialysis.
[9] Repeated trials, mechanical stirring during digestion or substitution of the supernatant of another commercial soybean meal did not change the values.
[10] Assay Protein C-1, Skidmore Enterprises, Cincinnati.
[11] Assay Protein C-1, Archer-Daniels-Midland, Minneapolis.
[12] Soybean protein, Nutritional Biochemicals Corporation, Cleveland.
[13] In further trials, addition of 0.8 gm of stable Zn at pH 3, as a water solution of $ZnSO_4$, increased ^{65}Zn dialysis from 1.6 ± 1% to 2.1 ± 0.5%, 4 mg to 4.4 ± 1% and 8 mg to 6.6 ± 0%.
[14] The whole pH 6.8 digest was used and the dialyzing water adjusted accordingly.

added. When the copper-containing pH 6.8 digest was dialyzed, the presence of copper decreased the passage of ^{65}Zn from $59 \pm 1\%$ to $7 \pm 1\%$. It appeared that copper had displaced zinc in the "carrier." The displaced zinc was then taken up by the phytate present, the next strongest binding agent, and as such was not dialyzable.

The zinc in the soybean meal supernatant was present in an organic complex and not as inorganic salt. The zinc was not precipitated as an insoluble phytate because of the presence of components which formed a more stable complex with zinc than phytate. The results also indicated that zinc was not freed by destruction of phytate during dialysis.

The meals and digestive fractions were analyzed for copper to determine whether a high copper content might account for appearance of zinc in a phytate complex due to occupation of a "carrier" of the sesame or safflower meals. Only traces of copper were found (table 2), indicating that "carrier" occupation was not operative with these meals.

Effect of soybean meal "carrier" on the dialyzability of the zinc of calcium·magnesium·zinc·phytate complexes. The capacity of the "carrier" to render soluble and dialyzable at pH 6.8 the zinc present in the insoluble calcium·magnesium complexes was measured. When the pH 6.8 precipitate to the sesame or safflower meals was mixed with the soybean meal supernatant, 15 to 29% of the zinc dialyzed to water (table 5). The supernatant from the equivalent of 1 g of soybean meal contained about 20 ppm of zinc already bound to the "carrier" from the soybean meal (table 1), and hence the "free" carrier had the capacity to render dialyzable an additional 12 to 20 ppm of zinc from the insoluble phytate complexes equivalent to 1 g of meal. The lesser amount of zinc rendered dialyzable from the safflower meal precipitate is thought to be due to the competition of the higher phytate content of the precipitate (table 1) with the carrier. Since the dialysates did not contain detectable amounts of phytate, the "carrier" apparently was complexed with the zinc rather than zinc phytate.

Soybean meal "carrier." Soybean meal contained a substance(s) which formed a more stable complex with zinc than did phytate. The complex was water-soluble and dialyzable. Continuing work shows the general nature of the carrier. Passage of the pH 6.8 supernatant through an anion exchange resin (Dowex 1, chloride form) did not remove the "carrier." When the supernatant was mixed with the pH 6.8 precipitate of 61 sesame meal, labeled with ^{65}Zn, $31 \pm 4\%$ of the ^{65}Zn dialyzed. Similarly, after passage through the resin and adjustment of the eluate to pH 6.8, $24 \pm 2\%$ of the ^{65}Zn dialyzed. Similar results were obtained when the supernatant or the eluate were added to the pH 6.8 precipitate of safflower or Venezuelan sesame meal. Phosphates or organic acids were not the active agents.

When the concentrated, purified, labeled dialysate from the labeled soybean pH 6.8 digest was chromatogramed on paper, the activity was associated with a spot which gave a positive ninhydrin reaction, presumably amino acids or a peptide. When a developing medium containing acid was used for paper chromatography, the activity was diffused and the presence of a sugar could be detected. Filtration of the concentrated dialysate through gels which separated substances up to a molecular weight of about 2000 (Sephadex 4 or Bio-Gel P2) did not result in a different pattern on subsequent paper chromatography. This suggests that the carrier is a fairly large molecule which contains a sugar moiety and amino acids as integral constituents.

"Carrier" capacity of isolated soy protein or basic amino acids. The "carrier" capacity of the soybean meal and that of each of 3 isolated soy proteins were compared. Isolated soy protein is widely used in zinc metabolism studies and a distinction between it and the protein of soybean meal is not always made.

One of the isolated soy proteins [14] produced a severe zinc deficiency in the chick, characterized by leg deformities and poor growth, when no zinc supplement was given (10). The addition of 15 ppm of zinc prevented the leg deformities and

[14] See footnote 7.

provided as good growth as 30 or 60 ppm. When this isolated soy protein was digested and the pH 6.8 supernatant added to the insoluble ^{65}Zn-labeled·phytate complex of the sesame meals (table 5) only about one-fourth as much ^{65}Zn appeared in the dialysate as with the soybean meal supernatant.

A second soy protein [15] required the addition of 30 ppm of zinc for good growth and prevention of leg deformities.[16] Very little dialysis of ^{65}Zn from the phytate complex of Texas 61 or Venezuelan meal occurred when the pH 6.8 supernatant was added (table 5).

A third "soybean protein"[17] had not been tested in low zinc diets. The pH 6.8 supernatant caused little dialysis of ^{65}Zn from the ^{65}Zn·labeled phytate complex of the sesame meals (table 5).

In the production of these isolated soy proteins the "carrier" components of soybean meal were decreased or lost.

The addition of histidine to isolated soy protein diets prevented some of the symptoms of zinc deficiency (17). Histidine is a strong chelator for many cations (14). The addition of L-histidine·HCl, L-arginine·HCl or L-lysine·HCl to the pH 6.8 precipitate of Texas 61 sesame or Venezuelan meals did not promote the dialysis of the bound zinc to water (table 5).

Dialysis of ^{65}Zn from labeled digests of animal proteins. Casein, egg white and fish meal were digested, labeled and the dialysis of ^{65}Zn measured. The object was to determine if phytate-containing vegetable proteins were unique in their zinc-binding and carrier properties or if such relationships might be found on in vitro digestion of animal proteins where phytate would not be involved. Zinc added to basal casein (2) or egg white diets (6) is available to the chick.

Only slight dialysis of ^{65}Zn occurred from a pH 6.8 digest of casein (table 5), with or without addition of pancreatin and erepsin. Increasing amounts of stable zinc were included to test the general binding capacity of the casein digest (table 5). When as much as 8 mg of stable zinc were added to the pH 3 digest equivalent to 10 g of casein, dialysis of ^{65}Zn was increased to only 6.6%. Chicks given casein rations which contained 1.6 mg of zinc per 22 g of casein utilized the zinc (2). As with the Venezuelan sesame meal, zinc could be bound in a non-dialyzable complex at one stage of digestion but be available in vivo. When the soybean meal "carrier" was added to the casein digest, 41% of the ^{65}Zn activity dialyzed (table 5). The pH 6.8 supernatant of an isolated soy protein [18] was only about one-sixth as active.

Little dialysis of ^{65}Zn occurred from the labeled digest or pH 6.8 precipitate of fish meal until the carrier was added (table 5). With an egg white digest, dialysis of about 14% of the ^{65}Zn occurred; addition of the "carrier" increased this to 66%.

The low dialyzability of the zinc of in vitro digests of the animal proteins and the capacity of the carrier to increase dialysis suggests that the added inorganic zinc was not "free" under the conditions of initial in vitro digestion.

The in vitro method was not designed to and did not measure the effect of several factors which could influence the in vivo utilization of zinc, that is, other ration ingredients or the possible action of the intestinal mucosa or blood, or of both, as transfer agents. However, a knowledge of the forms in which zinc may be present at intestinal pH can help explain the effect of other factors.

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Copper Deficiency in the Guinea Pig [1,2,3]

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ABSTRACT Copper deficiency was investigated in the guinea pig because this species resembles the lamb in that it undergoes considerable myelination in utero. When a semipurified pelleted ration containing 0.5–0.7 ppm copper was provided young female guinea pigs during growth and gestation a high incidence of ataxia, gross brain abnormalities and aneurisms was observed among the progeny. Agenesis of the cerebellum was one manifestation of defective nervous tissue development. Other animals showed gross changes only in the cerebral cortex tissue which was soft and translucent. Liver stores of copper proved to be within the range of values reported by others as characteristic of copper depletion in lambs known to have swayback.

Ataxia of young lambs associated with copper deficiency has been studied by several groups of investigators during the past 30 years. Variations of age of onset, type of symptoms and of the histopathology present in the brains of lambs suffering from swayback have been described. These manifestations of the disorder vary in different flocks of sheep and as a result, many questions relating to the pathogenesis of this syndrome remain unsolved. Areas which need further study include: the sequence of pathological changes which occur in brain tissues; the extent to which such pathology may be related to altered cytochrome oxidase activity; the relationship of defective elastin and other connective tissue components to vascular pathology in the brain; and finally the influence of genetic factors on the susceptibility of animals to deficiencies in dietary copper.

Innes and Shearer (1) were the first to make a comprehensive study of the disorder in the lamb. They characterized swayback as a demyelinating encephalopathy. Ataxic newborn lambs described by these investigators had massive collapsed areas in the cerebral hemispheres and showed shallow cerebral convolutions. Upon sectioning such tissue, large cavitations were observed. Behrens and Schulz (2, 3) proposed that the nervous tissue lesions of swayback resulted from venous stasis and demonstrated that edema and perivascular necrosis were present. Barlow and Butler (4–7) found cerebral lesions absent in approximately one half of the affected lambs examined, whereas cell necrosis and fiber degeneration occurred in the brain stem and spinal cord in 100% of the affected animals. Mills and Fell (8–10) studied lambs born to ewes receiving high intakes of sulfate and molybdate and described the tissue changes which they observed as follows: "Ataxia was accompanied by demyelination of the nuclei of the large motor neurones of the red nucleus in the brain stem, and by hypertrophy of oligodendroglia in the caudate nucleus." These investigators have suggested that there may be factors which, by influencing the distribution of copper between the brain and other tissues, are of importance in the etiology of swayback. Recently Roberts et al. (11, 12) have described cerebral edema in lambs with hypocuprosis. They consider this to be an acute delayed form a swayback. They did not observe cerebral cavitations but stated that: "Secondary brain changes attributable to pressure/anoxia included perivascular cuffing, cortical necrosis and cerebellar compressive effects. All affected lambs had the more chronic subclinical lesions of brain stem and cord neurones and of cord myelin typical of delayed swayback." Wiener (13) has introduced the import-

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[3] Presented in part at the 51st annual meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967.

ance of genetic factors on the occurrence of swayback in sheep.

Sheep have been unique among commonly used experimental animals in that they are the only species reported to show nervous tissue abnormalities at birth or during postnatal maturation of the nervous system in response to deficiencies in copper. We were interested in determining the response of guinea pigs to a low copper intake during fetal development since the guinea pig, like the sheep, undergoes considerable myelination during gestation (14, 15). In addition it was felt that the exceptionally complete intrauterine differentiation of nervous tissue components which occurs in this species should make the guinea pig a desirable small animal for use in other controlled studies of fetal morphogenesis and chemical differentiation.

EXPERIMENTAL

Guinea pigs of approximately 300-g weight were obtained from commercial sources and were fed a pelleted stock guinea pig diet [4] and lettuce for one week while they became acclimated. Following this they were fed a transition diet composed of one half stock pellets and one half purified diet. After 6 or 7 days the animals appeared to accept the semipurified diet satisfactorily. Early in our studies an attempt was made to use casein as the source of amino acids for a purified ration. Previous work using the guinea pig had indicated that a 30% level of casein was satisfactory for reproduction in this species (16). However, casein secured from several sources contained too much copper and identifiable moderate or severe deficiencies were not produced in guinea pigs under our laboratory conditions. Some attempts to increase the copper requirement by feeding sulfate or molybdate, or both, proved unsuccessful and were abandoned. Trials to determine whether feeding penicillamine would help clear the tissues of copper were eventually dropped.

A successful purified diet was achieved by modification of several dietary ingredients and an 8-kg quantity of pelleted feed was prepared as follows: (in grams) nonfat dry milk solids,[5] 4568; glucose,[6] 1320; roughage,[7] 960; salts,[8] 480; cottonseed oil, 320; potassium acetate, 200; magnesium oxide, 40; inositol, 16; choline chloride, 16; and vitamins.[9,10] A concentrate of vitamins A and D,[11] α-tocopheryl acetate, and vitamin B_{12} were added to the oil in the diet to achieve satisfactory distribution. The ingredients were combined and mixed with 540 ml of deionized distilled water. The material was pressed into the hopper of an electric meat grinder to produce pellets. The feed was spread thinly on a table to dry at room temperature. The dry ration was put into plastic bags and stored at $-4°$ until used. Plastic gloves were worn in the preparation and handling of all diets. Only deionized water was used for drinking purposes and for rinsing feed jars and cages. It was found that animals must be housed in stainless steel cages suspended in steel racks (or the equivalent) to reduce environmental contamination. Extreme care is necessary to avoid unexpected sources of copper. The copper content of the dry ration varied between 0.5 to 0.7 ppm. Control animals received 6 ppm copper furnished as copper sulfate. This was added to the salt mixture. It is important that the level of copper not exceed 0.7 ppm for moderate or severe deficiency symptoms.

Female animals were fed the low copper ration immediately after they became adjusted to the pelleted feed and were continued on the low copper intake during growth and throughout pregnancy. Male animals were routinely maintained with the stock diet and transferred to the low copper ration for several days before they

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

[5] Purchased from Golden State Sales Corporation, Burlingame, California. The product should be tested to secure milk solids of extremely low copper content.

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

[7] Alpha-cellulose purchased from Nutritional Biochemicals Corporation, Cleveland, was treated with EDTA and thoroughly rinsed with deionized distilled water to reduce the copper content.

[8] Salts in grams: $CaCO_3$, 300; K_2HPO_4, 325; NaCl, 168; $FeSO_4 \cdot 7H_2O$, 25; $MgSO_4 \cdot 7H_2O$, 28; KI, 0.4; $ZnCO_3$, 0.25; $MnSO_4$, 4.0. The salt mix used for the control animals contained in addition 0.30 g $CuSO_4 \cdot 5H_2O$.

[9] 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.

[10] 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}.

[11] Super D Drops, Upjohn Company, Kalamazoo, Michigan (synthetic vitamin A and vitamin D_3).

were put into the experimental cages for mating.

Rations and tissues for analysis were digested with a mixture of sulfuric acid, nitric acid, and perchloric acid and tissue copper content was determined by the carbamate colorimetric method (17).

RESULTS

When a semipurified diet providing 0.5 to 0.7 ppm copper was fed to young female guinea pigs, growth was satisfactory and reproduction was equal to that of females receiving the control diet. These adult females eventually showed mild anemia (Hb, 7.5–8.0/100 ml blood) and their hair coats became wiry and depigmented. In many cases 3 litters of young were produced by a given female fed the low copper diet over 8 to 10 months. These animals were, however, being housed in stainless steel cages which were suspended in galvinized racks and it is possible that the animals obtained some copper from these surfaces.

Litter size and birth weights of copper-deficient offspring agreed closely with control animals. Growth of the deficient young judged by weight gain began to slow up about day 12, and surviving copper-deficient animals of 50 or 60 days of age were markedly stunted (fig. 1). Newborn animals killed within 6 hours of birth provided tissues for determination of the phospholipid composition of brain and for histological examination.[12] The hair coat of the copper-deficient young continued on the experiment remained extremely soft and downy and became depigmented. A number of young died suddenly during the first postpartum month. Upon autopsy it was found that extensive intra-thoracic or intra-abdominal hemorrhage had occurred in these animals. Aneurisms were observed in young that were killed because they appeared to be nearly moribund. These occurred at single or multiple sites (fig. 2) and involved the aortic arch or the abdominal aorta between the celiac and renal arteries, or both of these. Determin-

[12] Everson, G. J., R. E. Shrader and Tong-In Wang 1968 Chemical and morphological changes in the brain of copper-deficient guinea pigs (manuscript in preparation).

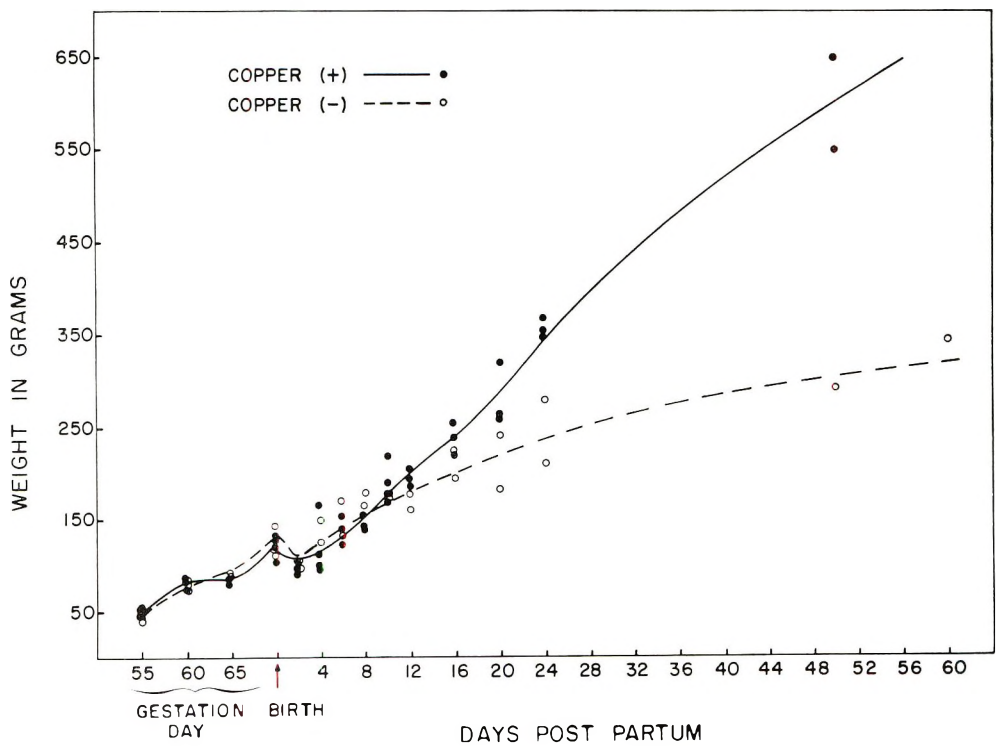

Fig. 1 Growth of offspring of guinea pigs receiving 0.5 to 0.7 or 6.0 ppm copper.

Fig. 2 Aneurysms at the aortic arch and in the abdominal aorta of a copper-deficient guinea pig 23 days of age.

ations of the elastin content of aorta were made and the amount of elastin was found to be markedly reduced in copper-deficient guinea pigs at 28 days of age.[13]

Lobulated kidneys were also observed at autopsy in copper-deficient newborn guinea pigs. The body and kidney weights of these animals were normal and no other indications of developmental immaturity were observed.

Abnormal behavioral patterns present in the most severely affected copper-deficient newborn guinea pigs included constant bobbing of the head, a swaying and unsteady gait, gross tremor of the body, and intermittent abnormal sounds. These disturbed animals failed to nurse or to eat and attempts to keep them alive by dropper feeding were usually unsuccessful.

Brain tissues of some of the copper-deficient guinea pigs appeared palely translucent, greyish in color or frankly hemor-

[13] Tsai, M. C., G. J. Everson and R. Shrader 1964 Copper deficiency in the guinea pig. Federation Proc., 23: 133 (abstract).

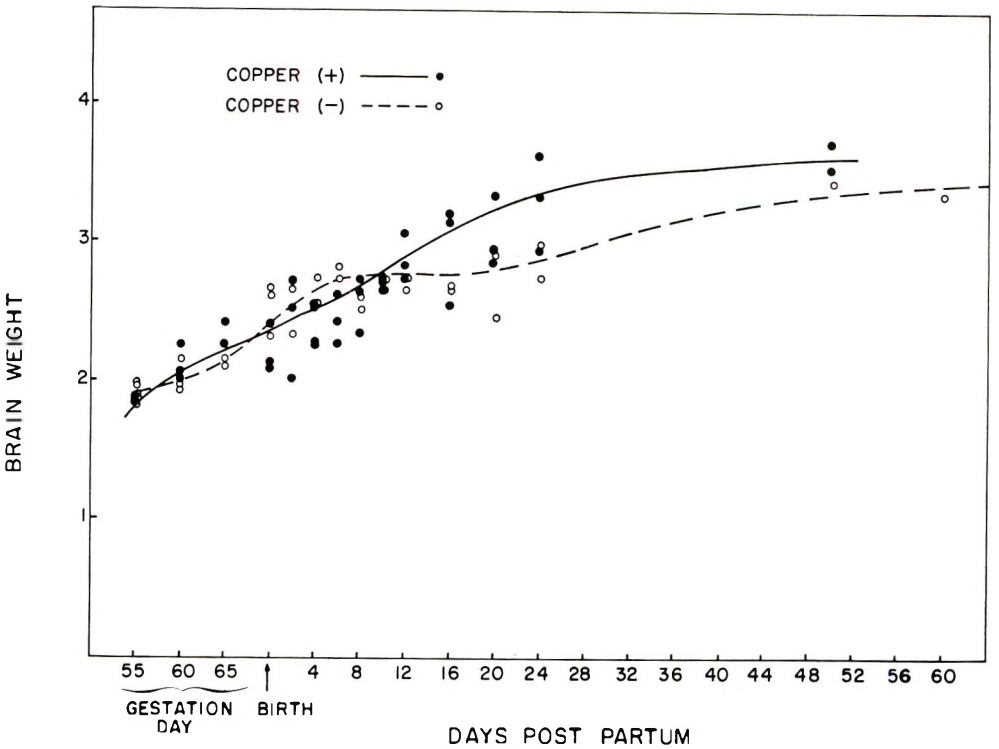

Fig. 3 Brain weights of copper-deficient and control guinea pigs.

rhagic. In several copper-deficient animals in which the cerebral tissue appeared edematous, brain weights were disproportionately high in relation to total body weight (fig. 3). Five of the animals that exhibited balance abnormalities at birth showed gross changes in cerebellar structure consisting of agenesis of the folia. Figure 4 illustrates these brain abnormalities. There is also a noticeable difference in the surface vascularity of the cerebral hemispheres of these brains. The width of the frontal cerebral hemispheres is narrowed and shows depressed areas over the anterior end of the third ventricle. The lateral ventricles were found to be distended, but did not show the confluent areas of cortical necrosis observed in deficient sheep. Cerebellar tissue is lacking except for the two small masses representing the paraflocculars and declival folia.

The copper content of liver tissue has been used to assess the degree of copper deficiency occurring in these animals. Table 1 gives these values. It will be noted that the offspring of control females had large amounts of copper stored in the liver at birth (145 μg/g of dry tissue). The total organ at birth contained approximately 300 μg of copper. At the next youngest age tested, 15 days, there was a decided decrease in the amount of copper present in the liver (45 μg/g of dry tissue), revealing that more than one half of the initial copper stores at birth had been mobilized to other tissues or had been excreted. The initial period of rapid decrease in copper reserves was followed by a more gradual change. Only 3% of the copper present in control animals at birth was present in the livers of deficient animals. There was essentially no change in the amount of copper in the total liver of deficient animals from birth to 40 days of age. The one animal examined at 63 days of age was apparently less severely affected by the deficiency regimen than the others, since its copper stores were less markedly depleted.

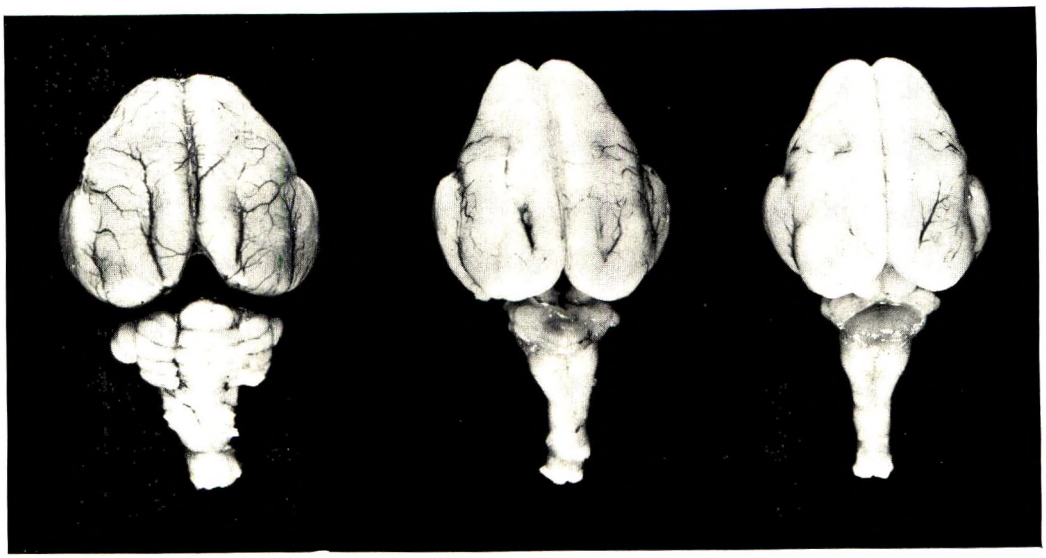

Figure 4A

Fig. 4 *A*. Dorsal view of the brain of a control guinea pig at birth (left) and 2 copper-deficient animals (right); *B*. medial view; and C. lateral view..

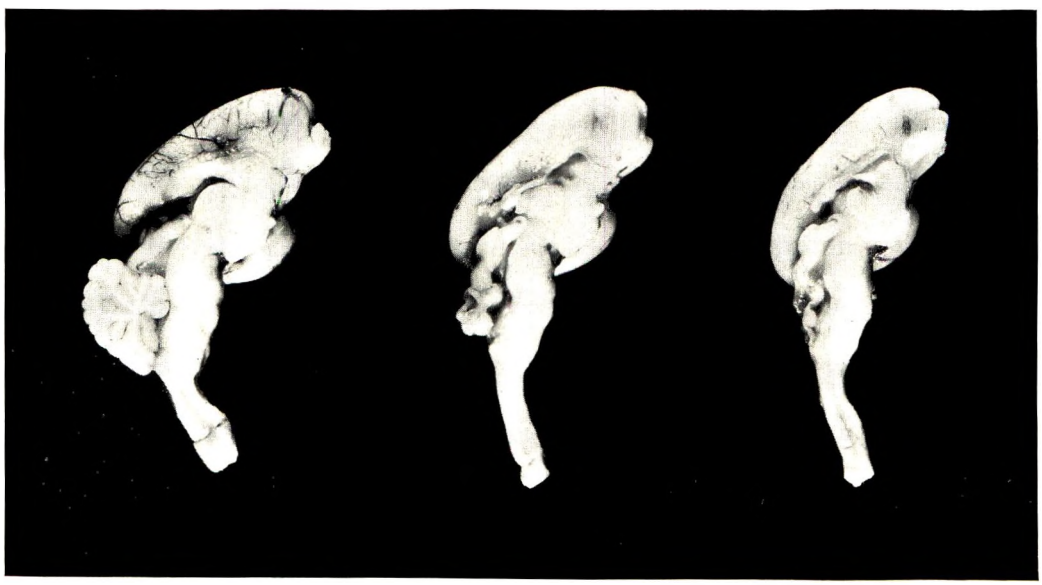

Figure 4B

DISCUSSION

A number of interesting avenues of research dealing with nervous tissue development can appropriately be undertaken using the guinea pig as the experimental animal. In utero the nervous tissue of this species undergoes rapid changes in myelination (18, 19, 15), in capillary density (20) and in growth of non-myelin brain constituents. As demonstrated by several investigators, oxygen uptake necessary for normal growth of the central nervous sys-

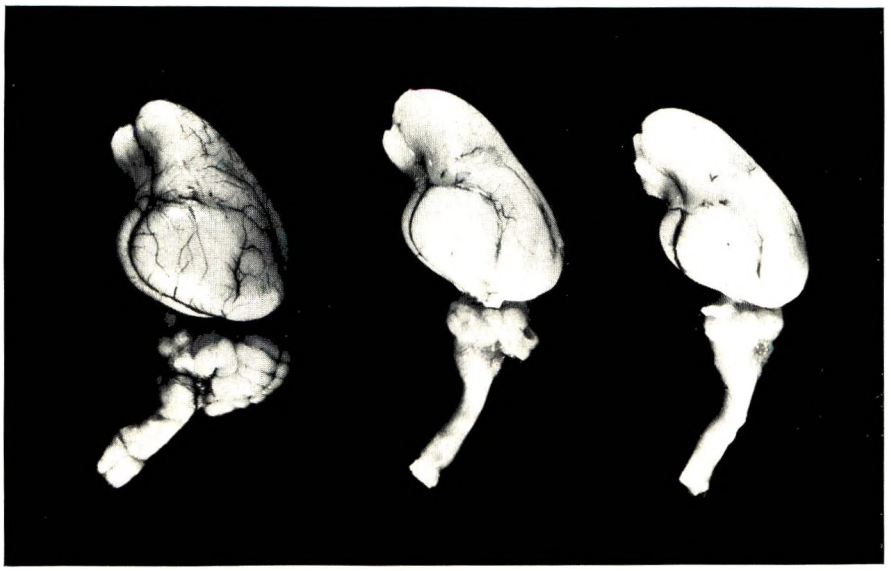

Figure 4C

TABLE 1

Copper content of livers

Ration	No. animals	Age	Liver		
			Wt	Copper content	
		days	*g*	*μg/g dry wt*	*μg/organ*
Without copper	5	newborn	5.9358	5.08	9.6
	1	15	6.1648	5.03	8.0
	2	23	5.6550	3.85	6.3
	5	28	7.5429	6.14	14.2
	1	40	4.2221	7.79	9.9
	1	63	9.8966	13.51	36.5
With copper	5	newborn	5.0204	145.91	297.7
	1	15	10.4811	45.48	133.0
	2	23	11.5243	37.50	96.5
	5	28	12.0618	35.81	115.2
	1	63	13.1874	20.75	78.0

tem of the lamb and guinea pig is especially critical in utero.

In the group of copper-deficient animals described in this report, copper reserves, based on the copper content of the liver, are very low, being approximately 3% of the amount transferred to the young of females having access to adequate amounts of the trace element. That control guinea pigs had drawn upon their original total liver reserves of copper to such an extent that they had reduced the total amount of copper present in the liver to approximately 50% during the first 15 days post partum, may mean that copper is rapidly being mobilized to maintain copper-dependent oxidases necessary for continued normal tissue synthesis. The available copper is undoubtedly being competed for for phospholipid synthesis through cytochrome oxidase activity and for elastin formation as described by Partridge (21), Hill (22, 23), Miller (24) and others. It is apparent that the deficient animals could not meet this need. More information is needed to determine the fate of this liver copper and factors which may regulate its mobilization.

In the deficient guinea pigs which show gross brain changes at birth, it can be

postulated that the supply of copper was too low during development to maintain the necessary cytochrome oxidase activity. The shortage of copper could in turn limit both the synthesis of phospholipids, as proposed by Gallagher (25) and restrict normal capillary density in the brain as proposed by Diemer and Henn (20). These possibilities are being explored in brain tissue by chemical investigations of phospholipid composition and histological studies of the brain.

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Influence of Periodicity of Eating on Energy Metabolism in the Rat [1]

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ABSTRACT Previous studies had shown that rats having access to food for a single daily 2-hour period (meal-fed) utilized their food for weight gain more efficiently than ad libitum-fed (nibbling) rats. This observation suggested that the energy expenditure of meal-fed animals was reduced. In the studies reported the spontaneous activity of meal-fed and nibbling rats was determined during the day (8 AM to 4:30 PM) and night (4:30 PM to 8 AM). Meal-fed animals had a significantly lower level of activity than the nibbling rats, particularly during the evening hours when activity was reduced by 57%. The basal oxygen consumption and heat production were not reduced in meal-fed animals. These data are interpreted as showing that the greater feed efficiency of the meal-fed rat is the result of a reduced activity level and consequently energy expenditure. The similarities between the obese human and meal-fed rat are discussed.

Rats having access to food for a short daily period (meal-fed) utilize their food for body weight gain more efficiently than do ad libitum-fed control rats (nibbling). Cohn et al. (1, 2) observed that rats force-fed an amount of food equivalent to that consumed by nibbling animals gained more weight and deposited more body fat than did the control rats. Leveille and Hanson (3) noted that meal-fed rats having access to food for a single daily 2-hour period gained weight at the same rate as nibbling control animals but consumed significantly less feed.

These observations, assuming similar absorption rates for meal-fed and nibbling rats, suggest that energy metabolism is strikingly altered as a consequence of meal-feeding. The increased body weight gain per unit of food consumed observed in meal-fed as compared with nibbling rats undoubtedly reflects a decreased energy expenditure. This in turn would be represented by a decreased metabolic rate or a reduced activity increment, or both. A decreased metabolic rate as a consequence of meal-feeding might be anticipated on the basis of the reported hypothyroidism induced in the rat by meal-eating (4).

The experiments to be reported were undertaken to determine whether energy expenditure is reduced by meal-feeding in the rat and if so whether the lowered energy expenditure is the result of a decreased metabolic rate or activity level. The results show that meal-feeding lowers the activity level but not the postabsorptive metabolic rate.

METHODS

Male rats of the Sprague-Dawley strain, weighing 300–350 g, were used. The animals were housed singly in cages having raised wire floors and in a temperature-regulated room (22°). The rats were fed a commercial diet [2] before the experiments. During the experimental period the animals were fed a purified diet having the following composition in g/100 g diet: vitamin-free casein, 18; glucose, 66.5; mineral mixture,[3] 4; vitamin mixture,[4] 2.2; fat,[5] 5; L-cystine, 0.3; and non-nutritive fiber, 4. The animals were divided into 2 groups, one of which was fed ad libitum (nibblers) and the other having access to food from 8 AM to 10 AM only (meal-eaters). Water was available at all times

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[1] This work was supported in part by Public Health Service Research Grant no. AM-10774-01 from the National Institute of Arthritis and Metabolic Diseases.
[2] Rockland Mouse/Rat Diet (complete), Teklad Inc., Monmouth, Illinois.
[3] The mineral mixture had the following percentage composition: NaCl, 10.81; $K_3C_6H_5O_7 \cdot H_2O$, 23.65; K_2HPO_4, 7.73; $CaHPO_4 \cdot 2H_2O$, 35.51; $CaCO_3$, 16.36; $MgCO_3$, 4.09; $FeC_6H_5O_7 \cdot 3H_2O$, 1.60; $CuSO_4 \cdot 5H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.14; KI, 0.004; and $ZnCO_3$, 0.04.
[4] For composition see Leveille (5).
[5] Crisco, Procter and Gamble, Cincinnati.

and food consumption and body weight were determined at weekly intervals. The animals were maintained on these treatments for at least 3 weeks, a period previously shown to be of sufficient duration to induce the meal-feeding response (6).

Spontaneous activity was determined in revolving cages connected to a counter which registered each revolution of the cage. The rats were allowed to adjust to these cages for one week before data collection was started. Activity was measured from 8 AM to 4:30 PM, and from 4:30 PM until 8 AM. Spontaneous activity was determined for 6 days and the value obtained for each rat was an average for the 6 days.

Respiratory quotient (RQ), oxygen consumption and basal heat production were determined using a Haldane respiration apparatus (7). Two trains were used and a meal-fed and a nibbling rat were run simultaneously to overcome any environmental factors which might influence the results. A 2-hour period was used for each determination and basal RQ's, oxygen consumption and heat production were determined between 6 AM and 8 AM, just before the meal-period. Nibbling rats were without food from 10 AM on the day preceding the determination; this therefore amounted to a period of 20 hours without food. The heat production was calculated from the CO_2 production since this was determined directly. The oxygen consumption and heat production data are expressed on the basis of metabolic body size ($W_{kg}^{0.75}$) to offset any possible effects of differences in body weight.

RESULTS

Rats, meal-fed or allowed to consume the experimental diet ad libitum, were used to study activity patterns. The animals were maintained on their respective feeding schedule for 30 days before testing. In table 1, data are presented on food consumption and changes in body weight which occurred during this period. The intervals indicated in the table were selected to illustrate the changes resulting from meal-feeding. During the first few days, food consumption was markedly reduced in the meal-fed rats, with a resultant loss in body weight. This was followed by a reduction in weight loss and finally a gain in body weight as food consumption increased. After 2 weeks, weight gain was

TABLE 1

Effect of meal-feeding on food consumption and body weight changes [1]

Period after start of experiment	Food consumption			Change in body wt		
	Meal-fed	Nibbling	*P* value [2]	Meal-fed	Nibblers	*P* value [2]
days	*g/rat/day*			*g/rat/day*		
1 through 3	7.0 ± 0.5 [3]	22.1 ± 1.4	< 0.01	−8.6 ± 0.5	1.6 ± 0.6	< 0.01
4 through 10	12.0 ± 0.8	22.3 ± 0.7	< 0.01	−2.5 ± 0.4	2.2 ± 0.1	< 0.01
11 through 14	13.2 ± 0.7	21.2 ± 0.7	< 0.01	6.5 ± 0.6	1.0 ± 0.3	< 0.01
15 through 30	16.6 ± 1.0	23.1 ± 1.0	< 0.01	1.0 ± 0.4	0 ± 0.6	ns

[1] Initial body weight (± SEM) was 326 ± 5 and 322 ± 7 for the meal-fed and nibbling animals respectively.
[2] Probability of difference (meal-fed vs. nibbling) being significant; ns = not significant.
[3] Mean ± SEM for 8 rats.

TABLE 2

Spontaneous activity of meal-fed and nibbling rats

Treatment	Body wt	Time-period studied		
		8 AM–4:30 PM	4:30 PM–8 AM	24 hr
	g	*rev/hr*	*rev/hr*	*rev/hr*
Meal-fed (8)	309 ± 3 [1]	35 ± 9	47 ± 9	43 ± 8
Nibbling (5)	344 ± 10	45 ± 8	110 ± 21	88 ± 15
P value [2]	< 0.01	ns	< 0.02	< 0.05
% change for meal-fed rats		−22	−57	−51

[1] Mean ± SEM for number of rats shown in parentheses.
[2] Probability of difference (meal-fed vs. nibbling) being significant; ns = not significant.

TABLE 3

Basal respiratory quotient (RQ), oxygen consumption and heat production of meal-fed and nibbling rats [1]

Exp. no.	Treatment	Body wt	RQ	O_2 consumption	Heat production
		g		$cm^3/hr/W_{kg}^{0.75}$	$kcal/hr/W_{kg}^{0.75}$
1	Meal-fed	358 ± 14 [2]	0.73 ± 0.02	882 ± 55	4.14 ± 0.24
	Nibbling	367 ± 5	0.74 ± 0.003	801 ± 41	3.78 ± 0.19
	P value [3]	ns	ns	ns	ns
2	Meal-fed	317 ± 14	0.71 ± 0.03	1059 ± 63	4.82 ± 0.16
	Nibbling	375 ± 10	0.70 ± 0.01	917 ± 58	4.19 ± 0.25
	P value	< 0.01	ns	ns	ns

[1] All rats were without food for 22 hours before starting the measurements.
[2] Mean for 4 rats ± SEM.
[3] Probability of differences being significant; ns = not significant.

TABLE 4

Respiratory quotient of meal-fed and nibbling rats at different times throughout the day

Treatment	Time after initiation of meal, hours [1]			
	2–4	6–8	11–13	22–24
Meal-fed	1.12 ± 0.03 [2]	1.09 ± 0.05	0.87 ± 0.004	0.73 ± 0.02
Nibblers	0.85 ± 0.03	0.86 ± 0.02	0.84 ± 0.05	0.90 ± 0.03

[1] Refers to meal-fed rats only; nibblers were fed ad libitum throughout.
[2] Mean for 4 rats ± SEM.

similar for meal-fed and nibbling rats although the meal-eating animals consumed significantly less food.

Following the 30-day adaptation to the feeding schedules, the activity patterns of meal-fed and nibbling rats were determined as shown in table 2. The 24-hour activity of meal-fed rats was decreased by approximately 50% as compared with nibbling control animals. This decrease was due largely to a reduced activity during the evening hours, the period of greatest activity for the control animals. Activity during the evening hours, as compared with values recorded during the day, was increased by 34% in the meal-fed rats which was in contrast with the 144% increase observed for the nibbling animals.

The studies summarized in table 3 were undertaken to determine the postabsorptive RQ, oxygen consumption and heat production of meal-fed and nibbling rats. Basal energy expenditure as judged from oxygen consumption and heat production were not significantly altered as a consequence of meal-feeding. There is a suggestion in both experiments of an increased postabsorptive energy expenditure in the meal-fed rats but this could not be established statistically.

The RQ of meal-fed rats was determined at selected times throughout the day as shown in table 4. The RQ's in excess of unity show that the meal-fed rat was synthesizing fat for at least 8 hours after the initiation of the daily meal. Twelve hours after the meal the RQ had dropped to 0.87, a value similar to that observed throughout the day for the nibbling animals, and demonstrating that about 50% of the calories were still being derived from carbohydrate. Before the daily meal, however, the RQ of the meal-fed rat had decreased to the fasting level, suggesting that over 90% of the calories were being derived from fat.

DISCUSSION

The data presented in table 1 are in general agreement with previous studies (1–3) showing that the efficiency of food utilization is increased in meal-fed rats. The data in table 1 show that, after 2 weeks of adaptation, meal-fed rats were able to maintain normal body weight gains for rats of this size (1–2 g/day) while consuming only about 70% of the amount of food consumed by ad libitum-fed controls. We have shown previously (3) that weight gain continues to be similar for meal-fed and nibbling animals through 16

weeks and that food consumption for meal-fed rats does not exceed 75% of that ingested by nibbling animals. The results of the present study show clearly that this greater feed efficiency is due to reduced activity and not to an alteration of the postabsorptive metabolic rate. This is somewhat unexpected in view of the report of Cohn et al. (4) suggesting that meal-feeding induces a hypothyroid condition. However, these findings are in agreement with the report of Fabry et al. (8) who noted a substantially lower oxygen consumption at night in intermittently starved rats.

The RQ's in excess of unity observed in meal-fed animals following the ingestion of the daily meal are in accord with the original observations of Tepperman et al. (9) and are indicative of active lipogenesis. This is in accord with the increased lipogenic capacity induced by meal-feeding as demonstrated both in vitro (3, 10–13) and in intact animals (14).

Some change in metabolic rate or activity pattern might have been anticipated to result from the reduced food ingestion in the meal-fed animals. However, inanition generally increases activity (15) and decreases basal metabolism (16) and neither of these effects was observed. This suggests that the decreased activity noted is, in fact, an effect of the single daily meal rather than an effect related to the amount of food consumed.

The significance of the present study with respect to the problem of obesity deserves consideration. As a consequence of the greater feed efficiency (1–3) and body fat deposition (1, 2) of meal-fed rats as compared with nibbling animals, meal-eating has been considered a possible etiological factor in human obesity. Such a consideration is strengthened by the observations that many obese individuals ingest most of their food in a short daily period (17–19). Also, Fabry et al. (20) have observed that human subjects ingesting frequent meals have a significantly lower incidence of obesity than individuals consuming fewer meals. Obese humans also have been found to ingest fewer calories than non-obese individuals (22–24). This apparent paradox is the result of the markedly reduced activity of the obese individual (21, 24, 25).

The meal-fed rat possesses the potential for obesity; it has developed an elevated lipogenic capacity (10–14) and, despite a reduced food intake, gains weight as rapidly as his nibbling counterpart (3). This increased efficiency is attained by reducing his activity and hence his energy expenditure. In many ways, therefore, the meal-fed rat resembles the obese human and may well serve as an excellent tool for the study of factors which induce and influence obesity.

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Diurnal Variations in Tissue Glycogen and Liver Weight of Meal-fed Rats [1]

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ABSTRACT The studies reported were designed 1) to describe the diurnal variations in tissue glycogen levels in meal-fed rats (animals having access to food for a single, daily, 2-hour meal), and 2) to compare the tissue glycogen storage capacity of meal-fed and nibbling (ad libitum-fed) rats. Rats trained to a meal-eating schedule and nibbling rats were maintained without food for 22 hours and then allowed access to food for 2 hours. The rats were killed at various times after the initiation of the meal, and liver, diaphragm and adipose tissue glycogen content was determined. Fasting liver glycogen levels were higher in meal-fed rats, but glycogen accumulated at the same rate in livers of meal-fed and nibbling rats for up to 8 hours after the start of the meal. Liver glycogen then decreased linearly from 8 to 22 hours. Meal-fed rats had a greater capacity to accumulate glycogen in diaphragm muscle and adipose tissue than nibbling rats. In meal-fed animals glycogen accumulated in diaphragm and adipose tissue for 8 hours, fell sharply between 8 and 10 hours after the initiation of the meal, and decreased more slowly from 10 to 22 hours. Liver weight increased significantly upon meal ingestion in both meal-fed and nibbling rats. Water accounted for about 66% of the increase and glycogen was responsible for the remainder. It was estimated that about 30% of the calories stored during and following the daily meal in meal-fed rats were in the form of glycogen. The data are discussed in terms of the importance of glycogen as a storage form of energy for the meal-fed animal.

Rats having access to food for a single short period daily (meal-fed) show marked alterations in a number of metabolic pathways. The meal-fed rat develops an increased lipogenic capacity (1–5), significant increases in the activity of several enzymes related to fatty acid synthesis (1, 2, 6, 7), and some apparent alterations in glycogen metabolism (1, 2, 8).

Tepperman and Tepperman (1) observed that liver glycogen of meal-fed rats was more resistant to depletion by fasting than that of control animals. This observation has been confirmed by other investigators (2, 8). Recently (8) it was shown that adipose tissue and diaphragm muscle of meal-fed rats accumulate glycogen more rapidly than tissue of control animals. This increased deposition of glycogen, which is in accord with the previous findings of Tepperman and Tepperman (1), was observed for up to 6 hours after the ingestion of food following a 22-hour fast.

The glycogen content of various tissues of meal-fed rats increases following the ingestion of a meal and must decrease preceding the next daily meal. However, the diurnal variations in tissue glycogen content of meal-eating rats have not been studied and consequently the rates at which these changes occur is unknown. A knowledge of the magnitude of glycogen storage and of its rate of deposition and depletion in meal-fed rats is of importance. Energy storage for use in the period between meals is of obvious necessity to the meal-eating animal. Lipid is apparently an important storage form of energy, since fat synthesis is extremely active in the meal-fed animal. Glycogen presumably also serves as a storage form of energy; however its significance is not known.

The present report describes the diurnal variations in tissue glycogen content of meal-fed rats. These studies also shed light on the significance of glycogen as a storage form of energy in the meal-eating animal. For purposes of comparison parallel experiments were also conducted with rats fed ad libitum. In the course of these experiments, it was noted that liver weight increased significantly following meal ingestion. An experiment is also reported showing that this increase in liver weight

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is due to an accumulation of water and glycogen, particularly the former.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used for all studies. Animals weighing between 306 and 355 g were used except for one study in which the initial body weight was 152 g. The average initial body weight for each experiment is indicated in the tables of results. The animals were housed individually in metal cages having raised wire floors and in a temperature-regulated room (22°). At the initiation of the study the animals were divided at random into 2 groups. One group was fed ad libitum (nibblers) and the other had access to food from 8 AM to 10 AM only (meal-eaters). In one experiment (exp. 2, table 1) the meal-eating rats were fed from 7 PM to 9 PM. Water was available at all times, and body weight and food consumption were determined at weekly intervals. The animals were fed a commercial rat diet [2] before initiation of the experiments. The composition of the diet fed during the experimental period has been described previously (9) and supplied approximately 19%, 12% and 70% of the calories as casein, corn oil and glucose, respectively. The animals were maintained on their respective feeding schedule for at least 3 weeks, a period shown to be sufficient to induce the lipogenic and enzymatic meal-feeding responses (8).

On the day preceding an experiment, food was removed from the nibbling rats at the termination of the meal period for the meal-fed animals. The animals were then treated as described in the tables of results. The rats were decapitated, and blood was collected. Pieces of liver and diaphragm muscle were rapidly removed, weighed and transferred to tubes containing hot 30% KOH; they were then digested in a boiling water bath for 30 minutes. Total liver weight was also recorded. Epididymal adipose tissue was weighed and transferred to tubes containing chloroform: methanol (2:1, v:v), and the lipids were

[2] Rockland Mouse/Rat Diet (complete), Teklad Inc., Monmouth, Illinois.

TABLE 1

Liver weight and glycogen content of liver, diaphragm and adipose tissue at varying times after meal ingestion in meal-fed and nibbling rats [1]

Time after initiation of meal	Regimen							
	Meal-eating				Nibbling			
	Liver wt	Glycogen			Liver wt	Glycogen		
		Liver	Diaphragm	Adipose tissue		Liver	Diaphragm	Adipose tissue
hours	*g/100 g body wt*	*mg/g*	*mg/g*	*μg/g*	*g/100 g body wt*	*mg/g*	*mg/g*	*μg/g*
				Experiment 1 [2]				
0	2.4 ± 0.1 [3]	22 ± 3	1.2 ± 0.4	43 ± 6	2.2 ± 0.1	4 ± 2	0.7 ± 0.3	15 ± 4
1	2.7 ± 0.04	26 ± 1	5.0 ± 0.4	141 ± 32	2.4 ± 0.1	17 ± 2	2.6 ± 0.2	20 ± 4
2	2.9 ± 0.04	43 ± 4	7.0 ± 1.0	386 ± 62	2.6 ± 0.1	24 ± 1	2.6 ± 0.1	14 ± 1
4	3.1 ± 0.1	66 ± 5	11.5 ± 1.2	1481 ± 446	2.8 ± 0.1	49 ± 2	5.3 ± 0.5	15 ± 1
6	3.4 ± 0.1	98 ± 3	14.5 ± 1.4	1604 ± 30	3.0 ± 0.1	65 ± 18	5.8 ± 0.5	29 ± 4
8	3.5 ± 0.1	116 ± 10	15.1 ± 1.1	2161 ± 335	2.9 ± 0.1	81 ± 8	6.1 ± 1.1	48 ± 12
				Experiment 2 [2]				
12	3.7 ± 0.1	111 ± 3	7.4 ± 0.9	607 ± 135	2.6 ± 0.1	52 ± 5	1.3 ± 0.4	33 ± 4
14	3.7 ± 0.04	100 ± 2	5.4 ± 0.2	179 ± 32	2.6 ± 0.1	45 ± 3	1.8 ± 0.3	31 ± 2
16	3.3 ± 0.1	77 ± 3	3.4 ± 0.3	63 ± 18	2.9 ± 0.1	48 ± 8	2.4 ± 0.2	27 ± 3
18	3.3 ± 0.1	72 ± 4	3.2 ± 0.5	60 ± 6	2.8 ± 0.1	50 ± 3	3.0 ± 0.3	26 ± 9
20	3.2 ± 0.04	55 ± 7	3.3 ± 0.3	48 ± 5	2.5 ± 0.04	33 ± 3	2.4 ± 0.3	19 ± 1
22	3.2 ± 0.04	46 ± 3	3.5 ± 0.2	53 ± 7	2.5 ± 0.03	22 ± 5	1.6 ± 0.2	27 ± 4

[1] All animals had been without food for 22 hours before initiating the experiment. One group was killed before feeding (0 time); another group was killed after having had access to food for one hour; all other groups had access to food for 2 hours and were killed at the times indicated after the start of the meal.

[2] The initial body weight for experiments 1 and 2, respectively, was 361 and 338 g. The rats used for both experiments were maintained on their respective treatments for 4 weeks; the final body weights (± SEM) were 371 ± 4 (exp. 1) and 323 ± 6 (exp. 2) for meal-fed rats and 416 ± 5 (exp. 1) and 387 ± 4 (exp. 2) for the nibblers.

[3] Mean for 5 rats ± SEM.

extracted. The defatted tissue was then digested in hot 30% KOH. Glycogen was coprecipitated with Na_2SO_4 from the KOH solution (10) and was quantitated by the anthrone reaction (11).

Serum free fatty acids were determined by the method of Dole and Meinertz (12), using nile blue A as the indicator. Liver moisture and nitrogen were determined by AOAC procedures (13), liver total lipids as previously described (14), and ash by oxidation with HNO_3 followed by H_2O_2. Statistical evaluation, where indicated, was carried out by means of Student's *t* test.

RESULTS

The data presented in table 1 show the changes observed in liver size and tissue glycogen content at various times after the initiation of the daily meal in both meal-fed and nibbling rats. Meal-fed and nibbling animals were maintained without food for 22 hours before the initiation of the experiment; they then had access to food for 2 hours and were killed at various times after the initiation of the feeding period, as indicated. The zero- and 1-hour groups were killed before they had been fed, and after having had access to food for 1 hour, respectively. Time periods from zero to 8 hours and from 12 to 22 hours were studied in separate experiments conducted at different times.

Liver weight was observed to increase in both meal-fed and nibbling rats for up to 8 hours after initiation of meal ingestion (table 1). From 12 to 22 hours there was a decrease in liver weight. The changes in liver size were paralleled by changes in glycogen content. However, the absolute increase in liver glycogen could not account for more than about one-third of the total increase observed. Liver glycogen increased up to 8 hours after the initiation of the meal in both meal-fed and nibbling animals. The meal-fed rats had a higher initial glycogen level and values were higher, throughout the 8-hour period, in liver of the meal-fed than in that of nibbling rats. However, the rates of increase during this period were similar. In the meal-fed rats the liver glycogen level at 12 hours after the initiation of meal ingestion was similar to that noted after 8 hours and decreased between 12 and 22 hours. In contrast with values for meal-fed rats, liver glycogen levels of nibbling animals had apparently decreased by about 36% between 8 and 12 hours after meal initiation. This apparent earlier and more rapid depletion of liver glycogen levels in nibbling as compared with meal-eating animals might well be related to the smaller meal ingested by nibbling rats (10 ± 0.4 vs. 16 ± 0.05 g/rat).

The data presented in table 1 also show the changes which occur in diaphragm and adipose tissue. Glycogen deposition in these 2 tissues during the first 8 hours after initiation of the meal was markedly higher in meal-fed as compared with nibbling rats. The level of glycogen in both diaphragm and adipose tissue of meal-fed rats decreased from 12 to 22 hours but was not markedly altered during this period in tissues of nibbling rats. It appeared that in both adipose tissue and diaphragm of meal-fed rats and in diaphragm of nibbling rats there was a marked reduction in glycogen levels from 8 to 12 hours after the initiation of the meal. However, it was difficult to determine with any degree of certainty the occurrence or the magnitude of these changes, since the 8- and 12-hour values were derived from different experiments conducted at different times.

The experiment summarized in table 2 was carried out to delineate the changes in liver weight and tissue glycogen levels which take place during the apparently critical period between 8 and 12 hours after the initiation of the daily meal. These data confirm the previous observation and demonstrate that the glycogen content of diaphragm muscle and adipose tissue of meal-fed rats is markedly reduced between 8 and 10 hours after the initiation of the daily meal. During this 2-hour period adipose tissue glycogen content decreased by 89% and that of diaphragm by 46% in meal-fed rats. The changes in tissues of the nibbling rat were not great and, at least for diaphragm, suggested that the levels had started to decrease before 8 hours. The large reduction in tissue glycogen content occurring during this period in meal-fed animals suggests that glycogen is being used as an energy source. To shed some light on the oxidative fuel available to the meal-fed rat during this period, serum free

TABLE 2

Liver weight and glycogen content of liver, diaphragm and adipose tissue at varying times after meal ingestion in meal-fed and nibbling rats [1]

Time after initiation of meal	Regimen									
	Meal-eating					Nibbling				
	Liver wt	Glycogen			Serum free fatty acids	Liver wt	Glycogen			Serum free fatty acids
		Liver	Diaphragm	Adipose tissue			Liver	Diaphragm	Adipose tissue	
hours	*g/100 g body wt*	*mg/g*	*mg/g*	*μg/g*	*μEq/liter*	*g/100 g body wt*	*mg/g*	*mg/g*	*μg/g*	*μEq/liter*
8	3.7 ± 0.1 [2]	93 ± 4	10.3 ± 1.4	2142 ± 510	200 ± 9	3.2 ± 0.1	66 ± 7	3.2 ± 0.4	21 ± 1	785 ± 123
10	3.6 ± 0.1	84 ± 4	5.6 ± 0.4	230 ± 68	355 ± 86	3.0 ± 0.1	61 ± 6	3.2 ± 0.6	21 ± 1	592 ± 118
12	3.6 ± 0.1	84 ± 5	6.0 ± 1.0	156 ± 107	355 ± 56	3.1 ± 0.2	57 ± 5	2.2 ± 0.4	22 ± 1	777 ± 66
14	3.5 ± 0.1	70 ± 6	3.7 ± 1.0	59 ± 10	918 ± 127	2.9 ± 0.1	52 ± 4	2.6 ± 0.3	26 ± 2	681 ± 34

[1] All animals had been without food for 22 hours before starting the experiment; they were then allowed access to food for 2 hours and killed at the times indicated. The rats were maintained on their respective dietary treatments for 4 weeks before use. The initial body weight was 355 g and the final body weights (± SEM) were 349 ± 6 (meal-eaters) and 403 ± 5 (nibblers).

[2] Mean for 5 rats ± SEM.

fatty acids were determined and these values are shown in table 2. Serum FFA levels were not elevated in the meal-fed rats until 14 hours after the initiation of the meal, implying that until that time carbohydrate was serving as the major energy source. In contrast with these observations in the meal-fed rats, serum FFA levels of nibbling animals were already elevated 8 hours after the initiation of the meal. The data presented in table 2 also confirm the decrease in liver weight occurring during this time period as noted in table 1 (exp. 2).

The rate of change in tissue glycogen level can be estimated from the data presented in tables 1 and 2. Such estimates are shown in table 3 for the time periods studied in experiments 1 and 2, table 1, and in table 2. The rate of change of tissue glycogen level was assumed to be linear for each of these time periods, and the equation for the best fitting line was calculated by the method of least squares. The values shown in the table are the slopes of these lines expressed as milligrams of glycogen gained or lost per gram of tissue per hour. These data show that, whereas the rates of change in liver of meal-fed rats were only slightly greater than those of the nibbling animals, the rates of change in tissue glycogen content were considerably greater

TABLE 3

Rate of change in glycogen content of liver, diaphragm and adipose tissue of meal-fed and nibbling rats at various times after meal ingestion

Tissue	Time after meal initiation [1]	Rate of change [1]	
		Meal-eating	Nibbling
	hours	*mg glycogen/g tissue/hr*	
Liver	0–8	+12.5	+9.7
	12–22	− 6.6	−2.6
	8–14	− 3.4	−2.3
Diaphragm	0–8	+ 1.74	+0.67
	12–22	− 0.37	+0.06
	8–14	− 0.97	−0.04
Adipose tissue	0–8	+ 0.282	+0.004
	12–22	− 0.045	−0.001
	8–14	− 0.316	+0.001

[1] Rate data were calculated by the method of least squares; the values presented are the slope of the least squares line. The data for the 0- to 8-hour period were calculated from experiment 1, table 1; for the 12- to 22-hour period, from experiment 2, table 1; and for the 8- to 14-hour period, from the data in table 3.

in diaphragm and particularly adipose tissue of the meal-fed animals.

The data presented in tables 1 and 2 suggest that adipose tissue and diaphragm muscle of meal-fed rats have a greater capacity to store glycogen than do similar tissues of nibbling animals and that the rate of storage is also much more rapid in the meal-fed rat. However, as pointed out earlier, the nibbling rats generally consume only about two-thirds of the amount ingested by meal-eaters during the 2-hour feeding period. To determine whether the observed changes were due to the difference in the amount of food consumed, the experiment summarized in table 4 was conducted. Meal-fed and nibbling rats maintained without food for 22 hours received 800 mg of glucose intraperitoneally; they were then killed at the times indicated, and liver and adipose tissue were taken for glycogen analysis. These data (table 4) show that, although the initial liver glycogen level was higher in the meal-fed animal, the rate of increase over the 2-hour period was similar for both meal-fed and nibbling rats (11 and 13 mg/g/hour for meal-eating and nibbling rats, respectively). In terms of the deposition of glycogen in adipose tissue, however, a considerable difference existed. Adipose tissue of meal-fed rats showed a 128% increase in glycogen level 30 minutes after glucose administration. Although the level decreased thereafter, it was still 39% above the fasting level 2 hours after glucose administration. In contrast, the glycogen content in adipose tissue of nibbling rats was not altered by the administration of glucose. These data show clearly that adipose tissue of meal-fed rats possesses a much greater capacity to store glycogen than does similar tissue of nibbling animals. Further, these data again confirm that the rates of increase in liver glycogen following glucose administration, whether orally or intraperitoneally, are similar for meal-fed and nibbling rats.

An increase in liver weight following meal ingestion was consistently observed in both meal-fed and nibbling rats. This increase was far greater than could be accounted for by the accumulation of glycogen. To determine which components were contributing to the increase in liver weight the experiment summarized in table 5 was conducted. Meal-eating and nibbling rats maintained without food for 22 hours were allowed access to food for 2 hours and were killed 6 hours after the start of the meal. The liver was removed and analyzed for water, protein, ash, lipid and glycogen. The results are shown in table 5. The data are expressed in terms of absolute amounts per 100 g of body weight and also as milligrams per gram of tissue. The liver weight increased by 41% in the meal-fed animals and 35% in nibbling rats following meal ingestion. The components analyzed for adequately accounted for the entire weight of the liver. All of the components determined increased when expressed per 100 g of body weight in both meal-fed and nibbling animals. Glycogen showed the greatest percentage increase, but quantitatively water was the component which showed the greatest increase. Water accounted for

TABLE 4

Adipose tissue and liver glycogen level of meal-fed and nibbling rats at varying times after the intraperitoneal administration of glucose [1]

Time after glucose administration	Liver glycogen		Adipose tissue glycogen	
	Meal-eating	Nibbling	Meal-eating	Nibbling
min	*mg/g*	*mg/g*	*μg/g*	*μg/g*
0	11 ± 1 [2]	2 ± 0.5	46 ± 2	13 ± 2
15	15 ± 7	5 ± 1	67 ± 27	22 ± 7
30	29 ± 5	17 ± 2	105 ± 14	17 ± 2
60	34 ± 3	18 ± 1	70 ± 9	19 ± 2
120	33 ± 4	28 ± 2	64 ± 12	17 ± 3

[1] All animals were without food for 22 hours before the initiation of the experiment; one group was killed before glucose administration (0 time); all other animals received 800 mg of glucose intraperitoneally in 2 ml cf saline and were then killed at the times indicated. The rats were maintained on their respective treatments for 3 weeks before use; the initial body weight was 306 g and the final weights (± SEM) were 296 ± 4 (meal-eating) and 363 ± 5 (nibbling).

[2] Mean for 5 rats ± SEM except the values for 120 minutes which represent the mean for 6 animals.

TABLE 5

Effect of meal ingestion on the composition of liver of meal-fed and nibbling rats [1]

Constituent	Meal-eating				Nibbling			
	Fasted	Refed	Difference	*P* value [2]	Fasted	Refed	Difference	*P* value
			%				%	
Liver wt, g/100 g body wt	2.90 ± 0.07 [3]	4.08 ± 0.04	+ 41	< 0.01	2.78 ± 0.05	3.76 ± 0.09	+ 35	< 0.01
Expressed as total liver content, mg/100 g body wt:								
Glycogen	51 ± 8	396 ± 10	+676	< 0.01	3 ± 0.4	273 ± 24	+9000	< 0.01
Protein	689 ± 13	724 ± 15	+ 5	ns	691 ± 13	735 ± 16	+ 6	ns
Ash	86 ± 2	95 ± 2	+ 10	< 0.02	82 ± 1	98 ± 3	+ 20	< 0.01
Water	2023 ± 48	2775 ± 37	+ 37	< 0.01	1934 ± 28	2592 ± 61	+ 34	< 0.01
Lipid	114 ± 5	139 ± 3	+ 22	< 0.01	117 ± 4	144 ± 5	+ 23	< 0.01
% of wt accounted for	102	101	—		102	102	—	
Expressed as mg/g liver:								
Glycogen	17 ± 2	97 ± 3	+471	< 0.01	1 ± 0.2	73 ± 7	+7200	< 0.01
Protein	238 ± 1	178 ± 3	− 25	< 0.01	249 ± 1	196 ± 4	− 21	< 0.01
Ash	30 ± 0.4	23 ± 0.5	− 23	< 0.01	30 ± 0.3	26 ± 0.4	− 13	< 0.01
Water	697 ± 1	681 ± 2	− 2	< 0.01	697 ± 1	690 ± 1	− 1	< 0.01
Lipid	39 ± 1	34 ± 0.7	− 13	< 0.01	42 ± 2	38 ± 1	− 10	ns

[1] All animals were without food for 22 hours before the start of the experiment. Half of the animals in each group were killed at this time (fasted); the remainder were allowed access to food for 2 hours and were killed 6 hours after the start of the meal (refed). The initial body weight was 152 g and the final weights (± SEM) 197 ± 4 (meal-eating) and 283 ± 2 (nibbling). The animals were maintained on their respective treatments for 3 weeks.
[2] Probability of differences being significant; ns = not significant.
[3] Mean for 7 rats ± SEM.

about 66% of the increase in liver weight in both meal-fed and nibbling rats. Glycogen accounted for 32% and 28% of the increase in liver weight of meal-fed and nibbling animals, respectively. A rather erroneous conclusion could be drawn by expressing the liver content as milligrams per gram of liver. As the data in table 5 show, with the exception of glycogen, all components decreased significantly when expressed as milligrams per gram of liver; yet there was an absolute increase in the amount of each component present. This is of course because the water content remained relatively constant per unit of liver weight. The data presented in table 4 do show that about 95% of the observed increase in liver weight resulting from meal ingestion is accounted for by the increase in glycogen and water. The remaining 5% is accounted for by smaller increases in protein, ash, and lipid in these livers.

DISCUSSION

The purpose of the present studies was to define the diurnal variations in glycogen content of various tissues of the meal-fed rat. For purposes of comparison, parallel studies were conducted with nibbling animals. The data presented show that liver glycogen increases linearly for about 8 hours after the initiation of the meal period in both meal-eating and nibbling animals and thereafter decreases in an almost linear fashion. The higher fasting level of glycogen noted in liver of meal-fed as compared with nibbling rats is in agreement with the original observation of Tepperman and Tepperman (1) and subsequent reports (2, 8). Tepperman and Tepperman (1) had previously shown that in meal-fed rats liver glycogen increases up to 5 hours after initiation of the daily meal and at 13.5 hours is somewhat reduced as compared with the 5-hour values. Leveille (8) has also observed increasing values up to 6 hours after feeding. The present report extends these observations by describing more completely the diurnal variations in liver glycogen level. It is also important to note that the results reported, as well as previous observations (8), illustrate that the rate of liver glycogen accumulation is similar for both meal-fed and nibbling animals (table 3).

In contrast with the observations relative to rate of glycogen deposition in liver, meal-fed rats accumulated glycogen in diaphragm and adipose tissue at rates higher than observed for tissues of nibbling rats. Adipose tissue in particular was capable of accumulating considerable quantities of glycogen in meal-fed rats, whereas virtually no accumulation occurred in tissue of nibbling animals. Chari-Briton et al. (15) and Leveille (8) have previously noted this enhanced capacity of adipose tissue of meal-fed rats to accumulate glycogen, and Tuerkischer and Wertheimer (16) and others (17) made similar observations for adipose tissues of fasted-refed rats. The present report extends these observations by showing that glycogen accumulation continues in these tissues for up to 8 hours after the initiation of the meal. The argument can be raised that the difference between meal-fed and nibbling rats in glycogen deposition in diaphragm and adipose tissue might be the result of the difference in food intake between these groups, since the nibbling rats consumed only about two-thirds of the amount ingested by the meal-fed animals. However, similar differences in muscle and adipose tissue glycogen content between nibbling and meal-fed animals have been observed when both groups were force-fed the same amount of diet (8). Since the meal-fed rat has been reported to have a higher rate of intestinal absorption than the nibbling animal (1), this study still leaves the possibility that the greater glycogen deposition in the meal-fed animals was the result of a greater and more rapid availability of glucose at the tissue level. In the present study equal amounts of glucose were administered to both groups intraperitoneally, which should have overcome the problem of differences in intestinal absorption rates. Yet the meal-fed rat still deposited considerable amounts of glycogen in adipose tissue, whereas the glycogen content of tissue from nibbling animals remained unaltered. Considering all these results, it seems rather clear that adipose tissue of meal-fed rats has developed the capacity to store glycogen, whereas tissue of nibbling animals lacks this capability. Similarly, the enhanced glycogen accumulation observed in diaphragm of meal-fed rats undoubt-

edly reflects an adaptation to the dietary regime.

The glycogen content of diaphragm muscle and adipose tissue of meal-fed rats decreases markedly between 8 to 10 hours after the initiation of the meal. This probably reflects the completion of intestinal absorption of carbohydrate and the use of tissue glycogen as an oxidative fuel. Judging from changes in serum FFA (table 2), carbohydrate serves, in large measure, as the substrate for oxidation until about 14 hours after the initiation of the meal. This is in accord with the respiratory quotient of 0.87 observed between 11 and 13 hours after the start of the meal period in meal-fed rats (9), which suggests that about 50% of the calories are still being derived from carbohydrate at this time. This implies that the storage of energy in the form of glycogen is sufficient to meet the meal-fed rat's requirements for 5 to 6 hours, if the postabsorptive state is reached within 8 to 9 hours after initiation of the meal. The basal energy requirement of the meal-fed rats used in experiment 1 (table 1) can be estimated at 53.1 kcal/rat/24 hours (9). Since the meal-fed rat has a reduced activity increment (9), the value of 6% of basal energy expenditure, reported for rats in a restricted environment (18), can be used as a fair estimate of the activity increment. This would increase the energy needs to 56.3 kcal/rat/24 hours or 0.632 kcal/hour/100 g body wt. (for meal-fed rats, exp. 1, table 1). The increase in body glycogen content of the meal-fed rats used in experiment 1 (table 1) during the 8 hours after the initiation of the meal was estimated using the values: liver weight, 3.5 g/100 g body weight (as measured); body adipose tissue content, 13 g/100 g body weight (19); muscle mass based on a muscle protein content of 9.75 g/100 g body weight (19) and a value of 75% H_2O in muscle, yielding a value of 39 g/100 g body weight for muscle mass. From these values, it was calculated that the meal-fed rats accumulated 923 mg of glycogen, equivalent to 3.692 kcal/100 g during the 8-hour period (during this same period of time the nibbling rats accumulated 437 mg of glycogen equivalent to 1.748 kcal/100 g body weight). This would meet the animal's energy requirements for 5.8 hours, a value close to the 5 to 6 hours assumed to be needed from the termination of meal absorption (8 to 9 hours) until fat mobilization occurs (14 hours). These calculations are obviously merely estimates; they do, however, make the point that the meal-fed rat does store substantial quantities of glycogen during and following the meal period.

In a similar manner it can be calculated that the meal-fed rats (exp. 1, table 1) ingesting 61.6 kcal (metabolizable energy) in their meal would have utilized about 18.8 kcal/rat during the 8-hour period following the start of the meal, while active energy storage was in progress. If this energy is assumed to be derived from the diet, 42.8 kcal/rat would have been stored as fat and carbohydrate. Of this, 13.7 kcal, or 32%, is estimated to be stored as glycogen and 68% as lipid, equivalent to 29.1 kcal or 3.2 g of lipid. Thus it can be seen that glycogen is an important storage form of energy in the meal-fed rat.

Adipose tissue shows the greatest relative adaptability to meal-feeding with respect to its ability to accumulate glycogen. However, quantitatively, the glycogen deposited in adipose tissue is equivalent to only about 3% of the total body glycogen which accumulates in the meal-fed rat following the daily meal. Therefore adipose tissue glycogen is apparently not of particular significance as a storage form of energy to be used for oxidative metabolism. Intuitively, it might be assumed that because of the relative magnitude of glycogen accretion in adipose tissue following a meal, an increase of several-hundred-fold (table 1), it must be of metabolic significance. Although its function is not understood, it is conceivable that glycogen is of importance in maintaining the unique metabolic functions of adipose tissue (20); however, such a conclusion must await further study.

The results reported for liver composition deserve comment. Increased liver weights have been observed upon refeeding of fasted rats (21–23). This increase appears to be largely a reflection of changes in water content (21). The results of the present study show that, in fact, water accounts for about two-thirds of the increase and glycogen the remaining one-third. Ash, pro-

tein and lipid also increase, but these components collectively account for only about 5% of the increase in liver weight. The data presented also illustrate how results expressed per unit weight can be misleading. Thus, liver lipid when expressed as milligrams per gram of liver decreased by about 10% as a consequence of meal ingestion, whereas when considered on an absolute basis the liver lipid actually increased by about 20%.

The results presented, showing the marked capacity of meal-fed rats to store glycogen during and following the meal-period, suggest significant alterations in the ability of the meal-fed animal to dispose of glucose. This is also suggested by the more rapid lipogenic capacity observed in meal-fed rats (5). Studies are in progress to describe the alterations in glucose metabolism induced by meal-feeding.

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Enhanced Severity of Experimental *Herpes simplex* Infection in Mice Fed a Protein-free Diet [1]

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ABSTRACT Four-week old weanling ICR male mice were made hypoproteinemic after feeding them an experimental protein-free diet for one week. Their capacity to respond to infection with *Herpes simplex* virus was compared with that of a group of mice fed a regular diet and whose serum protein content was normal. Mice fed the protein-free diet differed from the normal group in the following respects: 1) They had higher mortality, since a dose sufficient to kill all mice fed a protein-free diet in 9 days killed only 20% of the normal mice. 2) Although both groups developed viremia, the mice fed the protein-free diet had a 100-fold greater blood concentration of the virus; moreover, viremia persisted until death in the deficient mice, whereas it terminated in the normal ones between 36 and 42 hours. 3) The minimal dose sufficient to induce viremia in the mice fed the protein-free diet was tenfold lower than the one required to induce viremia in the normoproteinemic mice. 4) Mice fed the protein-free diet consistently developed encephalitis, whereas in normoproteinemic mice, encephalitis occurred only sporadically. Since both groups developed an interferon-like inhibitor in comparable quantities and neither group produced antibodies against *H. simplex* during the time these differences between the 2 groups were observed, failure of other defense mechanisms in the mice fed the protein-free diet must be responsible for the enhanced severity of *H. simplex* infection in them.

An understanding of the effects of hypoproteinemia on ability to resist infection is important for a rational approach to therapy of protein-calorie malnutrition. Infection constitutes a frequent and significant component of this syndrome (1), but the basis for the altered host response in hypoproteinemia is still unknown.

A deficiency of antibody response to typhoid antigen was demonstrated in hypoproteinemic adults by Wohl et al. (2). Similarly, Reddy and Srikantia (3) showed that, in children with kwashiorkor, the capacity to produce antibodies was directly proportional to the quantity of dietary protein consumed. Using the 17D yellow fever vaccine, Brown and Katz (4) showed that children with acute kwashiorkor failed to produce appropriate antibodies under the conditions that control children did. Similarly, the studies of Katz et al. (5) suggested a deficiency in antibody response to poliovirus type I vaccine in children with kwashiorkor.

Studies of animals, likewise, have suggested an impairment of host response to infection. Cannon et al. (6) showed that rabbits fed a protein-deficient diet synthesized antibodies against typhoid antigen at a rate much lower than the well-nourished controls; Wissler (7, 8) showed similar findings for pneumococcus in rabbits and rats. Dubos and Schaedler (9) showed that mice fed diets deficient in protein had an impaired capacity to resist bacterial infection. Likewise Sprunt and Flanigan (10) showed in 2 systems — swine influenza in mice and Rous sarcoma virus in chickens — that adult animals fed protein-deficient diets ultimately failed to resist viral infection. Ruebner and Bramhall (11) showed increased mortality of mice fed a diet deficient in protein, as compared with a normally nourished group, when both were infected with the same dose of a mouse hepatitis virus (MHV 3).

Our study was designed to examine the effect of feeding a protein-free diet to mice on their ability to resist viral infection. In this manner, we intended to produce in this animal model system conditions favorable to the development of kwashiorkor. Kwashiorkor may develop in well-nourished, breast-fed infants who are weaned

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and fed diets deficient in protein, although not necessarily deficient in other respects. We selected the *H. simplex* virus because its behavior in mice is well known, and because of its occasional pathogenicity for hypoproteinemic children (12).

MATERIALS AND METHODS

Male ICR mice were used. The protein-free diet,[2] and the regular diet [3] described in table 1, were administered in pellet form.

Virus. *Herpes simplex*, *H*-strain, originally isolated from a herpetic lip lesion and grown in primary rabbit kidney tissue culture for ten serial passages, was obtained from Dr. Tadasu Tokumaru of Children's Hospital of Philadelphia. The seed virus was passaged twice in WI-38 human diploid cell tissue culture. Its final titer was $10^{7.2}$ TCD_{50}.

Throughout the study, the virus was prepared and titrated in WI-38 human diploid cells. Cytopathic effect was taken as the end point and presumptive evidence of virus, which was then identified as *H. simplex* by neutralization with antiserum prepared in a guinea pig injected with stock *H. simplex*.

[2] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.
[3] Obtained from Taklad, Inc., Monmouth, Illinois.

TABLE 1
Composition of diets

	Regular diet	Protein-free diet
	g/100 g	*g/100 g*
Protein	24.69 [1]	0
Fat	6.53 [2]	10.0 [3]
Carbohydrate	55.67 [4]	70.0 [5]
Fiber (cellulose)	3.34	15.0
Vitamin mixture	0.28	0.45
Mineral mixture	0.39	0.39
Vitamin mixture, mg/100 g diet		
Thiamine	1.5	2.2
Riboflavin	0.7	2.2
Pyridoxine	1.0	2.2
Pantothenic acid	2.3	6.6
Vitamin D_2	1.0 (507 IU)	0.4 (220 IU)
Vitamin E (acetate)	96.0 (3.84 IU)	48.5 (1.94 IU)
Niacin	10.9	9.9
Choline	159.0	371.5
Folic acid	0.4	0.2
Vitamin B_{12}	0.003	0.3
Menadione	0.1	5.0
Vitamin A (palmitate)	2.8 (1385 IU)	4.0 (2000 IU)
Mineral mixture, mg/100 g diet		
Calcium carbonate	152.5	152.5
Cobaltous chlorate	0.0092	0.0092
Cupric sulfate	0.19	0.19
Ferrous sulfate	1.1	1.1
Magnesium sulfate (anh.)	22.9	22.9
Manganese sulfate	1.6	1.6
Potassium biphosphate	155.6	155.6
Potassium iodide	0.32	0.32
Sodium chloride	55.6	55.6
Zinc sulfate	0.22	0.22
Kilocalories/g of diet	3.41	4.22

[1] % of total protein: grain, 29.5; meat meal, 40.5; dried skim milk, 10.1; and fish meal, 20.2.
[2] % of total fat: semibleached tallow, 35; and vegetable oil, 65.
[3] Hydrogenated vegetable oil.
[4] Cane molasses.
[5] % of total carbohydrate: cornstarch, 22; and sugar cane, 78.

Antibody. Presence of antibody in the serum of mice was determined by successful neutralization of 100 TCD_{50} of the stock *H. simplex* by serially diluted serum, beginning with the undiluted and ending with a 1:8 dilution.

Serum interferon-like inhibitor. Titration of this inhibitor was carried out by the plaque reduction method in a mouse L cell tissue culture, using the Indiana strain of vesicular stomatitis virus (VSV) as the challenge. Sera of mice infected with *H. simplex* were exposed to ultraviolet radiation for 5 minutes to destroy any residual virus, and were dialyzed for 24 hours at 4° against an isotonic solution of pH 2, and then dialyzed against saline buffered to pH 7 for 24 hours at 4°. Following this, monolayers of L cells were exposed to serial dilutions of each serum for 18 hours and, after the serum was washed off, to 50 plaque-forming units (PFU) of VSV. Allowance was made for the presence of nonspecific inhibitors in the serum.

EXPERIMENTAL PROCEDURE

Four-week-old mice were fed the protein-free experimental diet at weaning and maintained with it during the entire study. The control mice were fed the regular laboratory diet. These diets were given ad libitum, and both groups of mice consumed them readily, although no attempt was made to assure that the intake was isocaloric. After one week, the mice fed the protein-free diet showed retarded growth (table 2), roughness of fur with hair that could be easily plucked, ascites, and reduced serum protein level (table 3). Mice from both groups were infected by intraperitoneal injection of 0.1 ml each of a suspension containing *H. simplex* virus; various concentrations were used. In addition, mice from each nutritional group were selected as controls and inoculated with only sterile diluent. Determinations of protein content, viremia, antibodies, and serum inhibitor were carried out on blood pooled from 6 mice. Determination of the virus in the brain was carried out on 10% suspensions of single brains.

TABLE 2
Weight of mice

Group	At time of weaning	After fed diets 1 week	
		Regular	Protein-free
	g	*g*	*g*
Control	17.4 ± 1.2 [1]	24.0 ± 1.1	
Experimental	17.4 ± 1.2		12.0 ± 1.4

[1] Mean ± SD.

TABLE 3
Serum protein level of mice

Group	Total protein	Albumin/globulin
	g/100 ml	*g/100 ml*
Protein-free diet	2.7	1.2/1.5
Regular diet	5.0	2.5/2.5

RESULTS

One hundred mice fed the protein-free diet and 60 mice fed the regular diet were injected with 150 TCD_{50} of *H. simplex.* Three days later deaths started occurring in the group fed the protein-free diet, whereas no deaths occurred in the other group until the sixth day. On the ninth day 100% of the mice fed the protein-free diet had died, whereas only 20% of the mice fed the regular diet had died by that time (fig. 1). The mice in both groups injected with sterile diluent were still alive at the termination of the experiment.

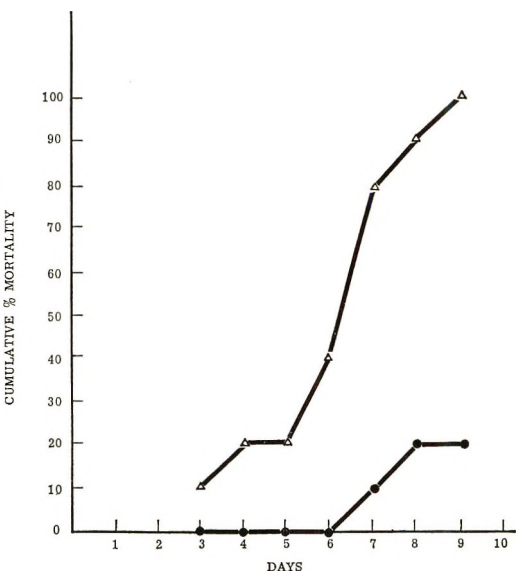

Fig. 1 Mortality rate in mice (100 fed protein-free diet; 60 fed regular diet) inoculated with 150 TCD_{50} of *Herpes simplex;* △———△ protein-free; ●———● regular protein.

TABLE 4

Viremia and antibody response

Dose [1]	Regular diet		Protein-free diet	
	Viremia	Antibody	Viremia	Antibody
3.2	+	+	+	?
2.2	−	+	+	?
1.2	−	−	−	?

[1] In $\log_{10}TCD_{50}$ titrated in WI-38 human diploid cells.

Viremia was demonstrated in both groups of mice (table 4). The lowest infective dose that resulted in viremia was $10^{2.2}$ TCD_{50} in the mice fed the protein-free diet, and in the control mice it was $10^{3.2}TCD_{50}$. However, well-nourished mice that received $10^{2.2}$ TCD_{50} of the virus exhibited antibody response on day 13 following injection of the virus, showing that they had been infected even though they showed no demonstrable viremia. None of the mice fed the protein-free diet survived beyond day 9, so that eventual determination of serum antibody could not be carried out in this group.

When mice in both groups were infected with $10^{4.2}$ TCD_{50} of *H. simplex*, virus was recovered from mice fed the regular diet 12 hours postinoculation and from mice fed the protein-free diet 24 hours postinoculation (table 5). The initial virus titer in the blood of mice fed the protein-free diet was $10^{3.3}$ TCD_{50}/ml. Among those fed the regular diet, the titer was $10^{1.5}$ TCD_{50}/ml, and remained unchanged until viremia terminated between 36 and 42 hours (table 6). The titer in the group fed the protein-free diet eventually rose to $10^{3.7}$ TCD_{50}/ml, with viremia persisting until death.

Virus was consistently recovered from the brains of the mice fed the protein-free diet beginning at 48 hours, but only sporadically among those fed the regular diet (table 5).

TABLE 6

Titer [1] of virus in blood after inoculation with $10^{4.2}TCD_{50}$

Group	Hours after inoculation					
	24	36	42	48	72	96
Protein-free diet	0	3.3	3.5	3.7	2.7	3.7
Regular diet	1.5	1.5	0	0	0	

[1] In $\log_{10}TCD_{50}$, titrated in WI-38 tissue culture.

When a dose of $10^{4.2}$ TCD_{50} was used, no antibodies against *H. simplex* could be detected in either group of mice throughout the 7 days following infection; by the seventh day all the mice fed the protein-free diet were dead. Antibodies were present in the blood of mice fed the regular diet on day 12 following infection. In no instance was virus recovered from either the blood or brains of the mice fed the protein-free diet and given no injection of the virus.

Ninety-six hours following infection, interferon-like inhibitor was demonstrated in the serum of both groups of mice given $10^{4.2}$ TCD_{50} of the virus. This substance resembled interferon in that it was not affected by a 24-hour exposure to pH 2 at 4°. Serum dilution of 1:4 in the mice fed the protein-free diet and 1:3 in the mice fed a regular diet resulted in a 50% reduction in plaques.

DISCUSSION

Our results suggest that protein-deficient mice cannot resist the acquired *H. simplex* infection as well as normal mice. An infective dose that killed only 20% of the control group, killed 100% of the mice fed the protein-free diet. This group had persistent viremia and died earlier than the well-nourished mice. Moreover, the concentration of virus was approximately 100-fold higher in the blood of mice fed the protein-free diet than in that of those fed

TABLE 5

Virus recovery from blood and brain after inoculation with $10^{4.2}TCD_{50}$ of Herpes simplex

Group	Source	Hours after infection															
		6	12	18	24	30	36	42	48	54	60	66	72	96	120	144	168
Protein-free diet	blood	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
	brain	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Regular diet	blood	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
	brain	−	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−

the regular diet. Encephalitis occurred in all protein-deficient mice given $10^{4.2}$ TCD_{50} of the virus, but was only sporadic in the corresponding group of well-nourished mice. During the period in which these differences were observed, both groups developed similar levels of interferon-like serum inhibitor; however, neither group produced antibodies against the infective virus. After the malnourished mice had died, antibodies did appear in the normal mice. The dose sufficient to induce viremia in the mice fed the protein-free diet was one-tenth that needed to produce viremia in the controls. Furthermore, this lower dose resulted in an inapparent infection of the control mice, since they ultimately exhibited antibody response.

Although the capacity of mice fed the protein-free diet to tolerate *H. simplex* infection was definitely lower than that of the controls fed a regular diet, the reasons for this difference are not clear. It is unlikely that it depends on the mechanism of antibody production or interferon synthesis, since no differences in either of these activities could be detected during our experiment.

This difference is analogous to that observed by Johnson (13) between suckling and adult mice in their susceptibility to *H. simplex*. He showed that mice developed with age a "barrier" against the spread of *H. simplex* to the central nervous system following extraneural inoculation and that this "barrier," which depended on macrophage action, was ineffective in the suckling mice. It is possible that hypoproteinemia leads to a breach of this barrier. We are evaluating this possibility.

Perhaps these differences do not result entirely from hypoproteinemia. Other deficiencies might have existed, although this is unlikely since an adequate quantity of minerals and vitamins was present in the diet. It is more difficult to interpret the influence of weight loss which may indicate inadequate caloric intake, or loss of muscle mass in response to hypoproteinemia, as was shown by Sprunt and Flanigan (10). Although one may postulate that this contributed to the enhancement of the severity of this viral infection, previous studies (14, 15) in which caloric deficiency was induced while protein content remained normal have shown that such animals resisted viral infection better than the well-nourished controls. We plan to conduct future studies to determine what influence *simultaneous* caloric and protein deficiencies may have on viral infection.

The present study has demonstrated an animal model system for a study of kwashiorkor in children. It can provide a basis for a more rigorous experimental approach to this disease, since any clinical analysis must necessarily be conducted while the children receive diets aimed at rapid repletion of their nutritional deficiencies.

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Free Amino Acids, Ammonia and Urea Concentrations in the Blood Plasma of Starved Lambs [1]

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ABSTRACT The effect of short periods of starvation on the free amino acid, ammonia and urea concentrations in blood plasma of Dorset Horn × Border Leicester-Merino lambs was studied in 3 age groups between 14 and 20 weeks of age. They were subjected to one of the following treatments: 1) fed a maintenance ration for 3 weeks; 2) fed a maintenance ration for 2 weeks and starved for 1 week; or 3) totally starved for 3 weeks. The concentration of free amino acids, ammonia and urea were determined in the blood plasma at the time of killing. Starvation for 3 weeks resulted in a significantly lower concentration of total α-amino nitrogen in the plasma when compared with the control fed lambs. The individual amino acids which were significantly lower were threonine, glutamic acid, alanine, valine, isoleucine and leucine and also urea. The concentration of lysine in the blood plasma was significantly higher in the starved lambs.

The effect of short periods of starvation (12–88 hours) upon the concentration of free amino acids in the blood plasma has been reported for numerous species. Hill and Olsen (1) reported that starving chickens for 48 hours resulted in increased concentrations of arginine, histidine, phenylalanine, glycine and tyrosine. However, Brown et al.[3] reported that steers fasted for 88 hours manifested increased concentrations of glycine, ornithine, lysine, valine, threonine, leucine, isoleucine and ammonia, whereas those of serine and alanine decreased.

Partial starvation for an extended period caused an overall decrease in the plasma concentration of free amino acids of children (2) and guinea pigs, (3). In both studies, the individual amino acids responded independently to starvation.

The present paper reports an experiment designed to study the effect of starvation, for 1 and 3 weeks, on the free amino acid, ammonia and urea concentrations in the blood plasma of lambs ranging in age from 14 to 20 weeks.

MATERIALS AND METHODS

All lambs were Dorset Horn × Border Leicester-Merino. At 14, 17 and 20 weeks of age 18, 27 and 18 lambs, respectively, were collected from the flock. They were shorn, brought into individual pens in the animal house and allowed 2 days for acclimatization before the commencement of the nutritional treatments. The 14-week-old animals were classified into 2 weight groups of 9 animals, 25 to 27 kg and 30 to 32 kg liveweight, the 17-week-old animals were classified into 3 groups of 9 animals, 25 to 27 kg, 30 to 32 kg and 34 to 36 kg, and the 20-week-old animals, 30 to 32 kg and 34 to 36 kg.

Three animals from each age and weight group were allotted at random to each of the following three treatments: 1) fed lucerne chaff ad libitum for 3 weeks; 2) fed lucerne chaff ad libitum for 2 weeks and subsequently starved for 1 week; and 3) starved for 3 weeks.

Liveweights were obtained at the commencement of the trial and immediately before slaughter at the conclusion of the trial. Empty body weights are expressed as the weight of the live animal immediately before slaughter minus the weight of the contents of the gastrointestinal tract.

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[3] Brown, H. E., H. O. Kunkel and J. M. Prescott 1961 Free amino acids of bovine plasma — identification and effect of fasting. J. Animal Sci., 20: 967 (abstract).

TABLE 1

Change in liveweight, the empty body weight and total α-amino nitrogen in the blood plasma with starvation

Wt at beginning of treatment	Age at beginning of treatment, weeks								
	14			17			20		
	Fed 3 weeks	Fed 2 weeks — starved 1 week	Starved 3 weeks	Fed 3 weeks	Fed 2 weeks — starved 1 week	Starved 3 weeks	Fed 3 weeks	Fed 2 weeks — starved 1 week	Starved 3 weeks
kg	% change in liveweight during treatment								
25–27	—	—	—	1.0	−23.2	−26.7	1.1	−14.6	−21.3
30–32	1.2	−14.0	−31.6	1.0	−16.8	−23.8	−0.3	−13.0	−21.9
34–36	1.3	−17.2	−20.2	1.1	−17.5	−32.3	—	—	—
	Empty body weight at conclusion of trial, kg								
25–27	—	—	—	28.2	23.3	21.0	28.2	26.0	21.7
30–32	27.5	23.4	18.0	26.9	22.0	19.3	26.0	20.3	20.0
34–36	25.0	19.2	16.7	22.7	17.4	15.5	—	—	—
	Total α-amino nitrogen in the blood plasma, mg/100 ml								
25–27	—	—	—	3.93	3.32	2.72	3.36	3.71	3.69
30–32	2.41	4.57	2.33	5.14	4.11	3.06	2.79	2.49	2.17
34–36	4.07	3.64	2.76	5.16	2.69	2.07	—	—	—

TABLE 2

Analysis of variance

	df	Mean squares						
		Taurine	Aspartic acid	Threonine	Serine	Glutamine	Glutamic acid	Glycine
Age	2	0.5846	0.1521	0.8282 *	0.0784	0.7197	0.0244	0.8072
Starvation	2	0.6992	0.0158	0.7106 *	0.5320	1.2698	3.7263 **	7.8034
Age × starvation	4	0.6081	0.0784	0.0647	0.1486	0.1006	0.4820	14.1667 *
Error	12	0.4297	0.0652	0.1732	0.2025	0.8467	0.2988	3.7115
Total	20							

	df	Mean squares						
		Alanine	Valine	Isoleucine	Tyrosine	Leucine	Phenyl-alanine	Ornithine
Age	2	0.4031	0.4031	0.0743	0.0127	0.6729	0.0061	0.0860
Starvation	2	2.0660 **	4.5569 *	1.0389 *	0.1855	2.1215 *	0.0192	0.1185
Age × starvation	4	1.3687 **	0.3925	0.1978	0.0731	0.3571	0.0412	0.0468
Error	12	0.1564	0.4797	0.2367	0.0635	0.6063	0.0375	0.0914
Total	20							

	df	Mean squares				df	Mean squares			
		Lysine	Histidine	Arginine	α-amino nitrogen		Ammonia	Urea	% wt change	Empty body wt
Age	2	0.6501	0.2187	0.2563	0.5391	2	0.6812	141.46	0.6945 *	1.359 *
Starvation	2	1.6744 *	0.0930	0.0185	2.4578 *	2	0.2125	2200.20 **	37.2945 **	14.042 **
Age × starvation	4	0.3758	0.1074	0.1715	1.1334	4	0.5040	123.54	0.5133 *	0.119
Error	12	0.3353	0.2709	0.1491	0.5396	54	0.3399	107.37	0.1630	0.327
Total	20									

* $P < 0.05$.
** $P < 0.01$.

Analytical. Blood samples were collected from the jugular vein at the time of slaughter and the plasma was deproteinized with sulphosalicylic acid as described by Hamilton (4). The levels of the free amino acids were determined using ion exchange chromatography (5), on a pooled sample of plasma of the 3 animals within each treatment. Ammonia and urea concentrations were determined on the plasma from each animal by the methods of Seligson and Seligson (6) and Van Slyke and Archibald (7), respectively.

RESULTS

Effect of dietary treatment upon body weight. The percentage change in liveweight and empty body weight for the lambs subjected to zero, 1 and 3 weeks of starvation are shown in table 1, and the analysis of variance in table 2.

The lambs fed ad libitum maintained their liveweight during the 3-week experiment, whereas those starved for 1 and 3 weeks lost an average of 17 and 25% of their initial weight, respectively.

There was a significant effect of age and a significant age × starvation interaction in the percentage change in body weight during treatment. This is due to the heavier and older lambs losing less weight relative to their initial body weight during the starvation treatment.

Effect of starvation on blood plasma concentrations of free amino acids, ammonia and urea. The average total free α-amino nitrogen concentration in the blood plasma of the lambs at the conclusion of the trial is shown in table 1. The average concentrations of the free amino acids, ammonia and urea are shown in figure 1. The analysis of variance is presented in table 2.

The total free α-amino nitrogen in the blood plasma of the lambs starved for 3 weeks was significantly lower ($P < 0.05$) than that of the control group. The individual amino acids which were lower in the animals starved for 3 weeks were threonine ($P < 0.05$), glutamic acid ($P < 0.01$), alanine ($P < 0.01$), valine ($P < 0.05$), isoleucine ($P < 0.05$) and leucine ($P < 0.05$). The concentrations of aspartic acid, serine, glutamine, glycine, tyrosine and phenylalanine were also lower but the differences were not significant.

After starvation for 1 week, the concentrations of threonine and glutamic acid in the blood plasma were significantly lower than in the control group, but with this shorter period of starvation the total α-amino nitrogen concentration was not significantly different from that for the fed lambs.

The lysine concentration in the blood plasma of the starved lambs was significantly higher than its concentration in lambs fed the maintenance ration. The concentration in the blood plasma of the lambs starved for 1 week was slightly higher than that observed in the lambs starved for 3 weeks. The concentration of ornithine in the blood plasma showed a similar trend but the differences were not significant. The concentrations of the other basic amino acids were not affected by the dietary treatments.

The concentration of urea in the blood plasma was significantly lower in the starved animals.

Effect of age and weight on the blood plasma concentrations of free amino acids, ammonia and urea. The analysis of variance shows that there was a significant effect ($P < 0.05$) of age on the concentration of threonine in the blood plasma. The concentration of threonine in the blood plasma was lower in the older animals. Total α-amino nitrogen, aspartic acid, glutamine, valine, isoleucine, leucine and phenylalanine concentrations were also lower in the older lambs but the effect was not significant.

There was a significant interaction of age and starvation for glycine and alanine. This is because starvation of the older and heavier lambs resulted in a smaller decrease in the concentrations of glycine and alanine with starvation compared with the control group.

Correlation between the concentrations of urea and ammonia in the blood plasma. There was a significant ($P < 0.05$) negative correlation ($r = -0.23$) between the concentrations of ammonia and urea in the blood plasma of all lambs.

Fig. 1 Effect of starvation and age on the free amino acids, ammonia and urea in the blood plasma of the sheep.

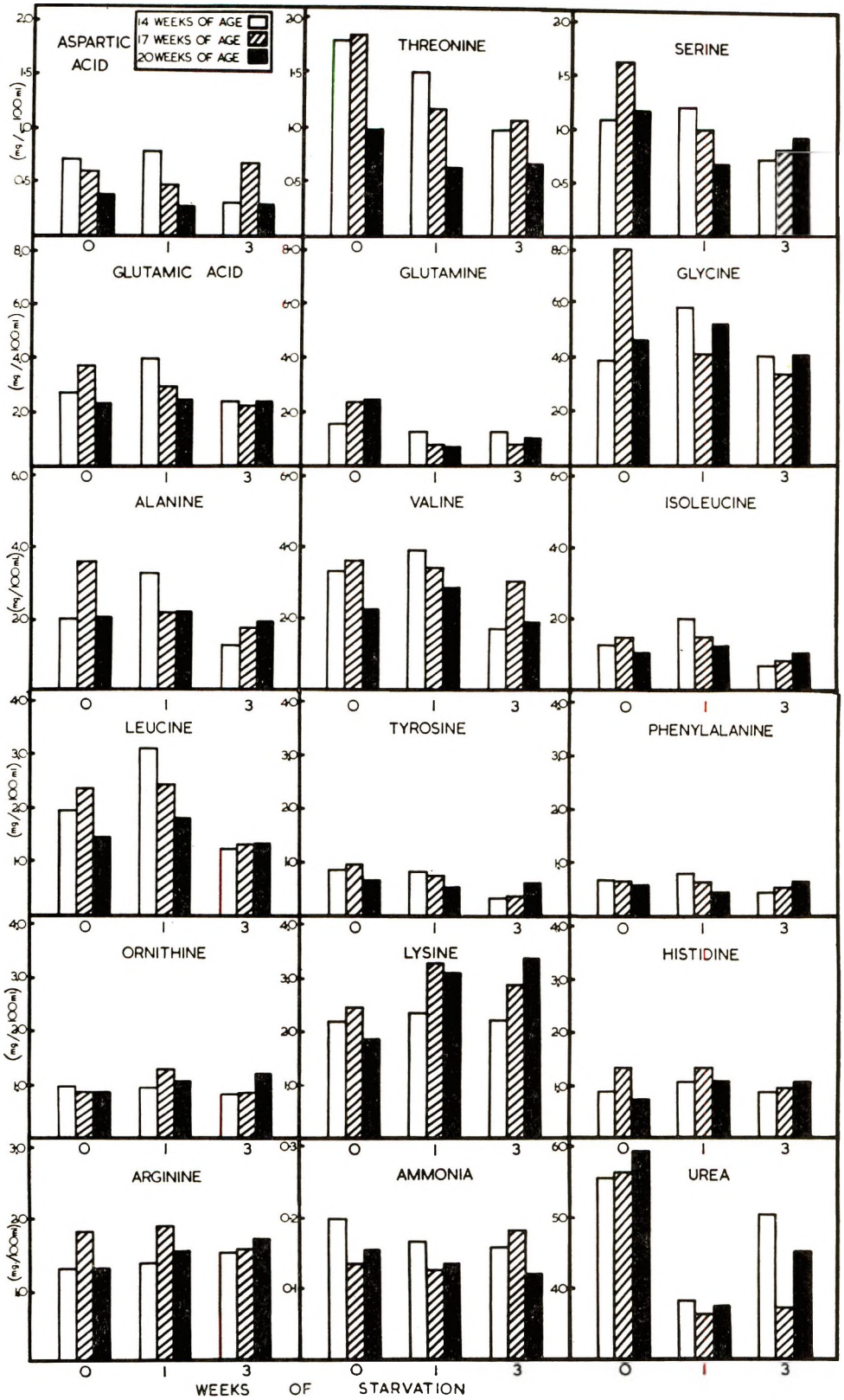
14 WEEKS OF AGE
17 WEEKS OF AGE
20 WEEKS OF AGE
ASPARTIC ACID
THREONINE
SERINE
GLUTAMIC ACID
GLUTAMINE
GLYCINE
ALANINE
VALINE
ISOLEUCINE
LEUCINE
TYROSINE
PHENYLALANINE
ORNITHINE
LYSINE
HISTIDINE
ARGININE
AMMONIA
UREA
(mg/100ml)
WEEKS OF STARVATION

Figure 1

DISCUSSION

The lambs used in this experiment were taken from a larger trial which was to measure the effect of starvation on carcass quality. It is for this reason that blood samples were not collected at the beginning of the experimental period to serve as a control within each group.

The present experiment shows a significantly lower concentration of free α-amino nitrogen in the blood plasma of starved lambs than in lambs fed a maintenance ration. The animals starved for 3 weeks had lower concentrations of free amino acids than those starved for 1 week. No animals were sampled after starvation for periods of less than 1 week; however, Brown et al.[4] showed a significant increase in the total concentration of free amino acids in the blood plasma of steers after an 88-hour fast. Although this could be a species difference it may be explained by the shorter period of starvation. Initially, during starvation the absorption from the digestive tract and breakdown of tissues may exceed the rate of utilization of amino acids as a source of energy. As a result there is an initial increase in the free amino acids in the blood plasma as observed by Brown et al.[5] followed by a decrease with the rapid utilization of amino acids as observed in the lambs starved for 3 weeks. Further experiments are required to verify this hypothesis.

Although starvation resulted in an increase in the total free amino acids, Brown et al.[6] showed a decrease in the concentrations of serine and alanine. In the present experiment the lambs starved for 3 weeks had significantly lower concentrations of free alanine and also valine, threonine, glutamic acid, leucine and isoleucine in the blood plasma than the control animals, but the difference for serine was not significant.

Gray et al. (8) and Hill and Olsen (1) showed that starving chickens for 48 hours resulted in a marked elevation in the concentration of lysine and threonine, whereas arginine, histidine, phenylalanine, glycine, tryptophan and tyrosine decreased. These results concur with those noted here for lambs with the exception that the threonine concentrations were lower in the starved lambs.

Large amounts of amino acids are released as a result of tissue breakdown during starvation, and they serve as sources of energy. The higher concentrations of free lysine in the blood plasma of starved sheep and chickens may be due to its slow rate of deamination and metabolism (9), while the lower concentrations of the other amino acids in the plasma may be due to their rate of utilization as energy sources, exceeding their rate of mobilization from the muscle tissue.

The lower concentrations of free amino acids in the starved lambs was less marked with the heavier and older animals, and this phenomenon may be due to the ability of the heavier lambs to use alternative sources of energy, such as stored lipids.

Arroyave et al. (2) noted that in the plasma of children suffering from marasmus, the concentrations of free alanine, valine, leucine, isoleucine, tyrosine, arginine, cystine, ornithine, glutamine and glutamic acid were low and the concentrations of taurine and aspartic acid were high when compared with those of normal children. There was no change in the ratio of "nonessential" to "essential" free amino acids. These trends observed in children generally agree with those observed for lambs subjected to 1 and 3 weeks of starvation, as the lambs showed no change in the ratio of "essential" to "nonessential" amino acids but in overall decrease in amino acids. An increase in the ratio of "nonessential" to "essential" free amino acids in the plasma is typical of children suffering from kwashiorkor (10).

The observed decrease in the concentration of urea in the blood plasma of lambs starved for 1 and 3 weeks when compared with the control fed animals, agrees with the observations of Lewis (11) who showed that urea levels were directly related to protein intake. The lower plasma urea concentrations will result in reduced excretion of nitrogen in the urine and reduced return of nitrogen to the rumen as urea in the saliva.

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[4] Brown, H. E., H. O. Kunkel and J. M. Prescott 1964 Ageing and fasting amino acid patterns in bovine plasma. Federation Proc., 23: 423 (abstract).

[5] See footnote 4.

[6] See footnote 3.

The ages of the lambs in the present experiment ranged from 14 to 20 weeks and they must be considered as ruminants. In a previous study, Leibholz (12) showed that the concentrations of free amino acids in the blood plasma of calves decreased as their age increased from zero to 24 weeks. For lambs, threonine was the only amino acid present in significantly lower concentrations in the older animals, but other amino acids showed a similar trend.

The negative correlation between the concentration of ammonia and urea in the blood plasma has been observed previously (13).

ACKNOWLEDGMENTS

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Lipid Transport in Experimental Dietary Hepatic Injury in Rats [1]

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ABSTRACT The effect of lipotropic factors on lipid transport was studied in rats fed hypolipotropic rations rich in protein (extracted peanut meal with or without added small amount of casein). With such a basal ration which permits at least initially satisfactory growth, the serum levels of cholesterol and lipoproteins fell precipitously. This was prevented by methionine and choline when mixed with the diet. However, given separately, the effect of methionine was not demonstrable and with choline, in general, significantly reduced. Vitamin B_{12} had not prevented an initial drop in the serum levels of cholesterol and lipoproteins, which gradually reverted toward the norm. Homocystine mixed with the diet, especially in combination with other lipotropic factors, produced significant hypercholesterolemia. Supplements of vitamin B_{12} greatly reduced in the liver the amount of total lipids with proportionate increase of the phospholipid fraction. In contrast, the total lipids were increased in the serum, together with phospholipids. Choline and to an even greater extent methionine, when fed separately were unable to prevent fatty liver in the long-term experiment. In the perirenal fat, in contrast with the lipids in the liver and in serum polyunsaturated fatty acids characteristic for cod liver were missing. A somewhat higher concentration of linoleic acid was found in the groups with supplements of choline and vitamin B_{12}.

Pure dietary hepatic injury is produced in rats fed hypolipotropic rations. Its chronologically first manifestation is fatty liver, followed after prolonged feeding — in 3 to 6 months — by fibrosis and finally cirrhosis of the liver. The amount of protein, the quality of the fat in the ration and the age of the rats may influence the severity of fatty infiltration and the consecutive development of cirrhosis of the liver (1–8).

Deposition of fat in liver has been related to the impaired production of phospholipids in the liver and in further consequence to the retarded transport of lipids from the liver to the blood (1, 2). Improved formation of phospholipids in the liver, followed by their increase in the serum, under the influence of choline (1, 8), was postulated on the basis of the increased ratio of those fatty acids in the liver and serum which are characteristic for phospholipids. In experiments of very short duration (10–14 days) this increase of phospholipids under the influence of choline as a supplement to a hypolipotropic diet was observed in female but not in male rats, (5–7). Young adult rats developed marked hypo-β (low density) lipoproteinemia when fed diets containing soy protein (Drackett protein) which is low in methionine and choline. These effects were prevented by the addition of 0.3% choline to the diet and partially prevented by casein, presumably because of its content of methionine (9). Similar changes were observed in rats fed a diet low in casein (10%) and in lipotropic factors (10, 11). Inasmuch as the β-lipoproteins of the serum contain about 75% of the total cholesterol (12, 13), it is not unexpected that in deficiency of lipotropic factors (choline, methionine and vitamin B_{12}), reduced serum cholesterol levels were observed (9, 14, 15).

These past studies were limited to short-term observations. The present studies

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were of longer duration, with special attention to the effect of vitamin B_{12} as well as other compounds involved in the metabolism of choline and methionine on transport of cholesterol and lipoproteins. Observations were also made on various lipid fractions and fatty acids in the serum, liver and depot (perirenal) fat as influenced by dietary factors.

EXPERIMENTAL METHODS

Male rats of the Sprague-Dawley strain were used. The average starting weight of each group of rats (10 animals) was 90 to 150 g in the various experiments, always identical in all groups of a given experiment. The composition of the basal rations is given in table 1.

Test substances in the first experiments were given as separate supplements (3 times weekly). In later tests they were usually mixed with the diet. In some cases the 2 methods of administration were compared. The dosage levels of the lipotropic and related substances tested have been discussed (16) and are indicated in the tables. In addition, a crude beef liver extract [5] and a purified liver extract (Ripason) [6] were used for comparison with vitamin B_{12}. The vitamin B_{12} content of the crude extract was 0.02 μg/mg, that of the purified preparation, 0.007 μg/ml. To avoid fatal cortical hemorrhagic necrosis of the kidneys, rats in the groups without any supplement or with supplements of cystine or homocystine received 10 mg of choline daily for the first 2 weeks of the experiment.

TABLE 1

Composition of basal rations

	EPM	EPM V	EPM VI
	%	%	%
Extracted peanut meal (EPM)	30	25	44
Casein (vitamin-test)	6	6	—
Sucrose	40	45	31
Salt mixture, USP XIV [1]	4	4	4
Lard	18	18	18
Cod liver oil	2	2	—
Vitamins A, E, and D in soya oil [2]	—	—	2
Vitamin mixture	— [3]	— [3]	1 [4]

[1] Zinc acetate (67.5 mg/100 g diet) added to correct for its absence in the salt mixture.
[2] Vitamin A, 1250 IU; *dl*-α-tocopheryl acetate, 10 mg; vitamin D, 180 IU; dissolved in 2 ml soya oil.
[3] An incomplete mixture of vitamins was given separately, 3 times weekly providing/day; (in micrograms) thiamine·HCl, 20; riboflavin, 25; pyridoxine·HCl, 20; Ca pantothenate, 100; menadione, 20; dissolved in 1 ml water. Niacin was incorporated in the diet, 10 mg/100 g.
[4] Contained: (in milligrams) thiamine·HCl, 0.5; riboflavin, 0.5; pyridoxine·HCl, 0.5; Ca pantothenate, 5; niacin, 8; *p*-aminobenzoic acid, 100; folic acid, 0.025; biotin, 0.02; ascorbic acid, 10; menadione, 0.5; inositol, 100; sucrose to make 1 g.

Rats were kept in separate cages. Feeding was ad libitum, with measured intake, except when specifically mentioned. The duration of the experiment was, in general, 140 days. Animals were fasted for 18 hours before blood samples were taken and before killing at the end of the experiment. Samples of serum, liver and adipose (perirenal) tissue were kept frozen until analysis was performed.

Extraction of serum lipids was accomplished according to the method of Sperry and Brand (17) modified by Bragdon (18). Total lipids were determined according to Bragdon (19), lipid phosphorus after Sunderman (20). An ultramicro modification of the method of Abell (21) using 25 μliters of serum was used for total cholesterol. Free cholesterol was determined with an ultramicro modification of the procedure described by Natelson (22). Serum lipoproteins were measured by a modification of the method described by Jenck et al. (23).[7] The values obtained are not absolute quantitative figures and serve only for comparative purposes.

The lipid fractions in the liver were determined in chloroform-methanol extracts (24). Phospholipids and free and total cholesterol were determined by the same methods as with serum.

Adipose tissue in approximately 5 mg samples was homogenized in a tissue grinder with 5 ml of chloroform-methanol (2:1, v:v).

For gas-liquid chromatography, aliquots of the chloroform-methanol extracts of liver, serum and adipose tissue containing 2 to 10 mg of lipids were evaporated to dryness under nitrogen. The fatty acids were converted into the methyl esters by refluxing the lipids with 5 ml of 5% H_2SO_4 in methanol for 4 hours at 70° in glass tubes with Teflon-lined caps. The mixture was then diluted with 5 ml of water and the methyl esters extracted with 3 succes-

[5] Wilson Company, Chicago.
[6] Robapharm, Inc., Basle, Switzerland.
[7] Spinco Division, Beckman Instruments Company, Tech. Bull. no. TB6043B.

sive 5-ml portions of hexane. The hexane extract was dehydrated over anhydrous sodium sulfate and then concentrated by evaporation under nitrogen.

The gas-liquid chromatography unit used was Barber-Colman model 10 with Sr 90 ionization detector. The identification of chromatographic peaks was based on comparison of the relative retention time of the methyl esters with those of standards.[8]

The fatty acids are reported according to the nomenclature proposed by Insull et al. (25). Standard error was not calculated for fatty acids that constituted less than 1% of the total because of the large relative errors in such values. The lard and cod liver oil used in the diet were analyzed and the composition of the mixture present in the food was calculated (table 2).

RESULTS

In the experiments of which the data are shown in tables 3–5, serum cholesterol and lipoprotein were measured at the beginning and at intervals up to 140 days. Control animals showed a sharp drop in the values for cholesterol and lipoproteins; these reduced levels persisted throughout the duration of the experiments. Choline chloride in a daily dose of 10 mg fed separately, did not prevent the abrupt and persistent decrease (table 3). In contrast,

[8] Purified highly unsaturated methyl esters were kindly supplied by the National Institutes of Health.

TABLE 2

Fatty acids in the fats used in the diet

	14:0	16:0	16:1	17:1 [1]	18:0	18:1	18:2	20:1 [2]	20:2 [1]	22:1	20:5	22:6	Total
Lard	1.2	25.0	1.6		6.8	57.0	7.8	0.5					99.9
Cod liver oil (CLO)	3.9	13.5	11.6	0.6	1.7	32.4	1.1	8.9	2.1	3.7	9.5	10.8	99.8
Lard + CLO (18:2, v/v) [3]	1.5	23.9	2.7	tr	6.3	54.5	7.1	1.3	0.2	0.4	1.0	1.1	100.0

[1] Tentative identification.
[2] Includes traces of 18:3.
[3] Fat mixture in the diet (calculated).

TABLE 3

Longitudinal studies on cholesterol and lipoprotein in the serum [1]

Days in exp.	Supplements [2]				
	None	Vitamin B_{12} in purified liver extract [3] (1 μg in 1 ml/day)	Crude beef liver extract [4] (20 mg/day)	Choline·Cl (10 mg/day)	Choline·Cl (25 mg/day)
	Cholesterol, mg/100 ml				
1	113 ± 6.0 [5]	112 ± 4.0	110 ±6.3	118 ± 6.8	116 ±4.9
15	50 ± 2.7	58 ± 3.1	60 ±2.7	59 ± 4.1	100 ±2.8
128	41 ± 1.2	93 ± 1.5	96 ±3.2	61 ± 2.5	82 ±3.2
	Lipoprotein, units				
1	108 ± 7.7	102 ± 3.6	95 ±5.1	110 ±10.3	100 ±4.8
15	31 ± 5.0	43 ± 7.4	39 ±4.4	37 ± 6.0	92 ±5.1
128	30 ± 2.3	83 ± 4.6	76 ±4.8	50 ± 6.8	65 ±4.7
Avg wt gain, g	165 ±10.7	308 ±13.3	326 ±7.5	222 ±12.6	267 ±9.5
Wt gain (g)/ 100 g diet	10.7± 0.6	14.1± 0.4	14.5±0.3	13.5± 0.3	13.1±0.4

[1] Diet EPM; average initial weight of rats, 91 g.
[2] Vitamin B_{12} in purified liver extract was injected subcutaneously; other supplements were administered orally.
[3] Ripason, Robapharm, Basle, Switzerland.
[4] Obtained from Wilson Company, Chicago.
[5] SE of mean.

TABLE 4

Effect of choline and of vitamin B_{12} with or without purified liver extract on serum cholesterol and lipoprotein [1]

Days in exp.	Supplements			
	None	Vitamin B_{12} (1 μg)	Vitamin B_{12} in purified liver extract [3] (1 μg in 1 ml) [2]	Choline·Cl (25 mg) [4]
	Cholesterol, mg/100 ml			
1	126 ± 4.7 [5]	112 ± 1.3	134 ± 2.0	127 ± 10.3
15	50 ± 3.0	49 ± 3.4	48 ± 2.2	87 ± 3.9
29	38 ± 3.8	49 ± 3.6	61 ± 3.8	58 ± 2.1
61	20 ± 1.6	78 ± 4.9	78 ± 3.4	52 ± 3.1
	Lipoprotein, units			
1	92 ± 9.1	113 ± 6.3	104 ± 4.2	111 ± 5.5
15	32 ± 3.7	36 ± 6.6	37 ± 2.6	62 ± 4.1
29	22 ± 2.1	35 ± 4.7	53 ± 4.6	43 ± 3.1
61	12 ± 1.9	66 ± 6.1	63 ± 4.6	46 ± 5.7

[1] Diet, EPM; average initial weight of rats, 113 g.
[2] Daily dose, injected subcutaneously.
[3] Ripason, Robapharm, Inc., Basle, Switzerland.
[4] Daily dose, oral.
[5] SE of mean.

TABLE 5

Dose-response relation of vitamin B_{12} to serum cholesterol and lipoprotein [1]

Days in exp.	vitamin B_{12}				
	None	0.03 μg [2]	0.1 μg	0.3 μg	1.0 μg
	Cholesterol, mg/100 ml				
1	120 ± 7.3 [3]	101 ± 7.0	126 ± 7.2	118 ± 6.5	116 ± 5.9
15	41 ± 1.9	43 ± 5.3	48 ± 4.6	62 ± 3.6	73 ± 6.8
29	36 ± 1.6	43 ± 3.2	54 ± 3.4	62 ± 3.9	77 ± 4.7
63	32 ± 3.3	49 ± 3.0	79 ± 3.2	93 ± 4.0	102 ± 4.4
	Lipoprotein, units				
1	117 ± 9.3	88 ± 6.0	111 ± 6.9	99 ± 7.0	95 ± 7.5
15	27 ± 5.7	21 ± 3.7	30 ± 3.0	46 ± 2.9	54 ± 6.7
29	24 ± 2.4	29 ± 2.2	43 ± 3.2	37 ± 1.6	49 ± 3.0
63	23 ± 2.3	37 ± 1.6	71 ± 4.9	78 ± 3.3	95 ± 5.2

[1] Diet EPM; average initial weight of rats, 97 g. For data on weight gain and food efficiency ratio, see table 4 (16).
[2] Daily dose, injected subcutaneously.
[3] SE of mean.

25 mg of choline chloride given separately, sustained initially normal serum levels of cholesterol and lipoprotein, followed by slow decline (tables 3 and 4). The pattern with crude beef liver extract, purified liver extract with vitamin B_{12} or vitamin B_{12} alone was quite different (tables 3 and 4): An early, significant decrease in the values of lipoprotein and cholesterol occurred but was succeeded by a gradual increase of levels. The mixture of purified liver extract and vitamin B_{12} was no more effective than vitamin B_{12} alone. Table 5 shows the correlation of the serum lipid values with the dose of vitamin B_{12}. The optimal dose may be calculated as between 0.3 and 1.0 μg. The initial drop was noticeable at all dose levels but was less marked with larger amounts of vitamin B_{12}.

The measured food intake showed either no significant or only slight differences within the groups of an experiment. For instance, in the first experiment discussed (table 3), the average daily intake in grams during the first 4 weeks was, for the first, second and fifth groups (control, vitamin B_{12} with purified liver extract, 25 mg choline·Cl) 10.7 ± 0.3, 11.5 ± 0.2 and 10.9

± 0.2. In another experiment (table 4), 4 groups of animals showed an intake of 10.2 ± 0.2 in the control group and slightly higher intakes with supplements (12.2 ± 0.3, 11.6 ± 0.3 and 12.5 ± 0.3).

In one experiment, supplements were mixed with the diet as well as given separately (table 6). The animals in the groups to be compared were strictly pair-fed with respect to amount of supplements. Methionine (at 0.3% level) was effective only when mixed with the diet but not when fed separately. Vitamin B_{12}, in the diet or fed separately, did not prevent an initial drop in the cholesterol levels, but, as in the earlier experiments, this was followed by a gradual rise towards normal values. Choline, at a level of 0.24% of the food maintained the serum cholesterol level within the normal range whether it was mixed with the diet or fed separately. The average daily intake of choline in these groups was 40 mg, thus far above the daily dose of 25 mg given in previous experiments. The weight gain in 20 weeks in the group fed choline mixed with the diet was 281.7(± 7.4) g, and in the group fed choline separately, 233.4 (± 3.7)g.

The comparison of the effect of various compounds involved in methylation and related metabolic reactions on serum cholesterol was studied in several experiments (tables 7 and 8). The basal ration was EPM VI and the supplements, singly and in combination, were mixed with it. Cholesterol was determined at the end of the 140-day experiment. As expected, the cholesterol of the control group was very low; with supplements of choline, methionine and vitamin B_{12} the values were normal. The choline concentration was only 0.13%. At this level the diet provided an average of about 20 mg/day of choline, less than the amount (25 mg) which fed separately was found ineffective in maintaining normal serum levels (table 3 and 4). Dimethylethanolamine and homocystine also prevented a decrease in the level of cholesterol. Unexpectedly, homocystine, in several instances when given alone, but especially in combination with lipotropic factors produced levels which were, in general, significantly higher than in any other groups.[9] To illustrate this point more clearly, cholesterol values in tables 7 and 8 have been compared statistically with the figures for homocystine plus vitamin B_{12}. Cystine behaved quite differently, having no effect on serum cholesterol.

The effect of choline, methionine and vitamin B_{12}, as long-term supplements to a hypolipotropic diet, on lipids of liver, serum and adipose tissue are shown in tables 9 and 10. These animals were fed choline and methionine as separate supplements; vitamin B_{12} was injected subcutaneously.

Highest values for serum cholesterol occurred in the group receiving vitamin B_{12}, the lowest in the control group; values for the groups fed choline and methionine were intermediate: significantly different from both control and vitamin B_{12}-treated groups (table 9). The proportion of the

[9] Two animals of group 5, table 6 had levels of 263 and 268 mg/100 ml, respectively.

TABLE 6

Effect of choline, methionine and vitamin B_{12} given in the diet or separately on serum cholesterol [1]

Days in exp.	None	Supplements [2]					
		Choline		Methionine		Vitamin B_{12}	
		Mixed in diet	Given separately	Mixed in diet	Given separately	Mixed in diet	Given separately
		mg cholesterol/100 ml serum		*mg cholesterol/100 ml serum*			
1	138 ± 5.4 [3]	140 ± 7.1	140 ± 3.2	151 ± 6.3	141 ± 5.5	127 ± 2.9	133 ± 4.9
32	51 ± 6.6	88 ± 3.1	88 ± 6.8	89 ± 7.2	52 ± 6.9	56 ± 4.3	62 ± 4.6
57	25 ± 3.2	87 ± 6.8	81 ± 3.9	79 ± 3.6	33 ± 2.8	89 ± 4.2	77 ± 6.8
113	38 ± 2.2	92 ± 2.4	93 ± 5.2	86 ± 5.2	40 ± 3.4	99 ± 2.5	97 ± 4.8
140	38 ± 1.9	91 ± 2.6	93 ± 2.9	102 ± 4.5	45 ± 3.7	99 ± 2.4	98 ± 5.4

[1] Basal diet, EPM VI; average initial weight of rats, 107 g. For data on weight gain and food efficiency ratio, see table 6 (16).
[2] Amount of supplement per 100 g of diet: choline-Cl 0.24 g; methionine, 0.3 g; vitamin B_{12}, 5 μg. Pairs of groups received the same supplement in the diet or separately were pair-fed with respect to the supplement.
[3] SE of mean.

TABLE 7

Effect of vitamin B_{12}, methionine, choline, homocystine and dimethylethanolamine on serum cholesterol [1]

Group	Supplements [2]	Cholesterol	*P* value
		mg/100 ml	
1	None	54 ± 2.1 [3]	< 0.01
2	Vitamin B_{12}	128 ± 4.7	< 0.01
3	Methioine (M)	140 ± 4.8	< 0.01
4	M + vitamin B_{12}	130 ± 8.0	< 0.01
5	Homocystine (H)	160 ± 21.7	ns
6	H + vitamin B_{12}	148 ± 7.9	< 0.05
7	Dimethylethanol-amine (D)	93 ± 5.7	< 0.01
8	D + vitamin B_{12}	113 ± 4.7	< 0.01
9	D + M	120 ± 5.8	< 0.01
10	D + M + vitamin B_{12}	134 ± 4.1	< 0.01
11	D + H	148 ± 9.7	< 0.05
12	D + H + vitamin B_{12}	171 ± 6.0	ns
13	Choline·Cl (C)	136 ± 10.4	< 0.01
14	C + vitamin B_{12}	136 ± 6.4	< 0.01
15	C + H	153 ± 7.8	< 0.05
16	C + H + vitamin B_{12}	182 ± 11.3	control [4]
17	C + M	139 ± 6.8	< 0.01
18	C + M + vitamin B_{12}	143 ± 1.2	< 0.01

[1] Diet EPM VI; average initial weight of rats, 105 g; tested after 140 days. For weight gain and FER see table 7 (16).
[2] Supplements were mixed with the diet in the following concentrations: DL-methionine, 0.6%; DL-homocystine, 0.69%; choline·Cl, 0.13%; dimethylethanolamine, 0.085%; and vitamin B_{12}, 10 μg/100 g.
[3] SE of mean.
[4] The highest average cholesterol value was used as a baseline for statistical comparison in order to make the significantly high serum cholesterol in the groups receiving homocystine clearly evident.

TABLE 8

Effect of vitamin B_{12}, choline, cystine, homocystine and methionine on serum cholesterol [1]

Group	Supplements [2]	Cholesterol	*P* value
		mg/100 ml	
1	None	34 ± 1.6 [3]	< 0.01
2	Vitamin B_{12}	99 ± 3.3	< 0.01
3	Choline·Cl (C)	94 ± 3.2	< 0.01
4	C + vitamin B_{12}	100 ± 4.4	< 0.01
5	Cystine	39 ± 2.0	< 0.01
6	Cystine + C	92 ± 2.5	< 0.01
7	Cystine + C + vitamin B_{12}	95 ± 2.4	< 0.01
8	Cystine + vitamin B_{12}	102 ± 4.0	< 0.01
9	Homocystine (H)	90 ± 3.6	< 0.01
10	H + C	154 ± 6.2	ns
11	H + C + vitamin B_{12}	148 ± 3.8	ns
12	H + vitamin B_{12}	148 ± 9.8	control [4]
13	Methionine (M) + C	124 ± 3.9	< 0.01
14	M + C + vitamin B_{12}	109 ± 5.1	< 0.01

[1] Diet EPM VI; average initial weight of rats, 139 g; tested after 140 days.
[2] Supplements were mixed with the diet in the following concentrations: DL-methionine, 0.6%; choline·Cl, 0.13%; homocystine, 1.08%; cystine, 0.49%; vitamin B_{12}, 10 μg/100 g. For weight gain and FER see table 8 (16).
[3] SE of mean.
[4] See footnote 3 to table 7.

cholesterol present as ester was approximately the same in all of the treated groups and significantly higher than in the control group. Total lipids and phospholipids of the vitamin B_{12}-treated animals were also significantly higher than those of the other groups. The values for animals given choline and methionine did not differ from those of the control group (table 9).

In the liver (table 10), in reversal of the observations for serum, the lowest val-

TABLE 9

Lipids in the serum [1]

Group	Supplement [2]	Total lipid	Phospho-lipids	Cholesterol			
				Total	Free	Ester	
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*% of total*
1	None	162 ± 14.5 [3]	69 ± 5.9	48 ± 2.4	20 ± 3.2	28 ± 2.0	58 ± 5.0
2	Vitamin B_{12}	263 ± 14.5	94 ± 5.8	81 ± 2.1	17 ± 1.9	64 ± 2.6	79 ± 1.6
3	Choline·Cl	175 ± 12.2	76 ± 6.7	56 ± 2.1	12 ± 0.9	44 ± 1.9	78 ± 1.5
4	Methionine	151 ± 10.2	63 ± 7.2	58 ± 1.5	14 ± 0.9	44 ± 1.9	76 ± 2.0

[1] Animals given diet EPM V for 140 days; average control starting weight, 96 g.
[2] DL-Methionine, 25 mg daily and choline·Cl 25 mg daily, as separate supplements, vitamin B_{12} 1 μg daily by subcutaneous injection.
[3] SE of mean.

TABLE 10

Lipids in the liver [1] *(fresh tissue)*

Group	Supplement [2]	Total lipid	Phospho-lipids	Cholesterol			
				Total	Free	Ester	
		mg/100 g	*mg/100 g*	*mg/100 g*	*mg/100 g*	*mg/100 g*	*% of total*
1	None	19.1 ± 2.45 [3]	2.2 ± 0.09	1156 ± 63	337 ± 71	819 ± 71	71 ± 3.3
2	Vitamin B_{12}	8.0 ± 0.96	3.3 ± 0.13	820 ± 95	255 ± 71	565 ± 82	69 ± 2.6
3	Choline·Cl	26.5 ± 0.98	2.7 ± 0.11	1358 ± 52	247 ± 17	1111 ± 51	82 ± 1.3
4	Methionine	28.0 ± 0.95	2.4 ± 0.13	1195 ± 36	247 ± 17	951 ± 40	79 ± 1.6

[1,2] See footnotes for table 9.
[3] SE of mean.

ues for total lipids, cholesterol, cholesterol esters occurred in group 2 receiving vitamin B_{12}. In contrast, and thus in analogy to the results for serum, the phospholipid fraction in the liver was higher on group 2 than in any other group (table 11). Expressed in percentage, the phospholipid in the liver of rats treated with vitamin B_{12} amounted to 41% of the total lipids, whereas for the other groups the figure was from 8 to 12%.

Fatty acids in the liver (table 11) as already indicated by the figure for total lipid, were highest in the groups receiving choline and methionine as separate supplements (22.3 and 23.7 g, respectively, in 100 g of fresh liver), lower in the control rats (16.1 g) and at an approximately normal level in the animals treated with vitamin B_{12} (6.0 g). Expressed in percentage of total fatty acids the liver of the vitamin B_{12} group contained a significantly higher amount of arachidonic, palmitic and stearic acids and a smaller amount of oleic and high polyunsaturated fatty acids than the other groups.

In the serum the most significant differences among the groups were in the unsaturated fatty acids (table 11). Arachidonic acid was higher in all of the treated groups than in the controls. Linoleic acid was increased in the vitamin B_{12}- and choline-treated groups, but not in those receiving methionine. Except for oleic acid, which was even lower in the groups receiving choline and methionine than in the controls, values of these acids for the choline and methionine groups were between those of the vitamin B_{12} groups and the controls.

In adipose (perirenal) tissue there was an identical pattern of fatty acids in the groups with supplements of vitamin B_{12} and choline (table 11). As compared with these groups, in the control and methionine treated rats there was an increase in the percentage of palmitic and palmitoleic acids and a decrease in oleic and linoleic acids.

DISCUSSION

As known from the relevant literature (9, 14), choline and methionine when mixed with a hypolipotropic basal diet were found to be effective in preventing the drop in the serum level of total lipids, lipoprotein, phospholipids and cholesterol. In the present studies this effect was less marked or even absent when choline and methionine were fed separately. The results are analogous to those found (in the same ani-

TABLE 11

Fatty acid composition of serum, liver and depot lipids [1,2]

Group	Supplement	16:0	16:1	18:0	18:1	18:2	20:4	Higher PUFA's
				% of total fatty acids				
				Serum				
1	—	31.8 ± 1.00 [3]	2.4 ± 0.2	13.0 ± 0.46	22.0 ± 0.75	12.1 ± 0.28	11.5 ± 0.58	5.6 ± 1.00
2	Vitamin B_{12}	25.6 ± 0.85	1.2 ± 0.06	10.3 ± 0.35	24.9 ± 0.89	14.1 ± 0.34	14.3 ± 1.12	8.7 ± 1.21
3	Choline	27.7 ± 0.69	1.6 ± 0.11	13.3 ± 0.41	20.0 ± 0.53	14.3 ± 0.21	14.1 ± 0.62	7.1 ± 0.75
4	Methionine	29.3 ± 0.60	2.0 ± 0.13	14.4 ± 0.58	19.3 ± 0.50	12.9 ± 0.31	14.1 ± 0.36	6.4 ± 0.78
				Liver				
1	—	29.3 ± 1.27	1.0	5.5 ± 0.45	41.5 ± 1.59	9.2 ± 0.33	3.0 ± 0.25	9.5 ± 0.83
2	Vitamin B_{12}	34.2 ± 1.27	0.9	9.0 ± 1.57	34.0 ± 2.89	9.9 ± 0.69	6.2 ± 1.07	5.2 ± 0.60
3	Choline	25.0 ± 0.78	1.0	3.4 ± 0.21	46.3 ± 1.11	11.9 ± 0.43	1.8 ± 0.10	9.7 ± 0.66
4	Methionine	24.9 ± 0.78	1.1	3.9 ± 0.14	44.4 ± 1.10	11.6 ± 0.65	2.6 ± 0.16	10.4 ± 1.19
				Perirenal fat				
1	—	31.3 ± 0.97	5.1 ± 0.48	2.8 ± 0.16	54.2 ± 1.11	5.1 ± 0.41		
2	Vitamin B_{12}	25.8 ± 1.16	3.4 ± 0.37	2.8 ± 0.19	39.9 ± 1.10	6.8 ± 0.43		
3	Choline	25.1 ± 0.60	3.0 ± 0.18	3.0 ± 0.15	61.1 ± 0.79	6.6 ± 0.26		
4	Methionine	30.1 ± 1.10	4.4 ± 0.44	2.5 ± 0.14	56.5 ± 1.14	5.1 ± 0.30		

[1] Only fatty acids found in measurable quantity were considered.
[2] All supplements were given separately.
[3] SE of mean.

mals) for growth, food efficiency ratio, liver fat and to some extent, especially with methionine given separately, also to the formation of ceroid with cirrhosis (16). The decrease of lipids and their fractions in the serum might be related to the accumulation of fat in the liver without compensating transport to the periphery. The analytical findings for total lipids, phospholipids and cholesterol in the liver (table 10) are in full accord with this assumption. However, as for other parameters or metabolic injury, the question why these compounds could not act as efficiently as lipotropic factors when fed separately as when mixed with the diet is unanswered. Preferential decomposition by intestinal bacteria when they were given separately and in high concentration was a suggested explanation (16).

The action of vitamin B_{12} differed from that of choline and methionine in 2 respects. Its effect was in general the same regardless of mode of administration: in the diet, fed separately or injected subcutaneously. The second finding, hitherto unreported, was that whereas choline and to a lesser extent also methionine, even when fed separately, maintained at least initially, normal levels for cholesterol and lipoprotein in the serum, vitamin B_{12} did not prevent an initial decrease in the values which was followed by a gradual increase toward the normal range. This appears to indicate, in accord with previous observations (16), that vitamin B_{12} is not directly, but only secondarily, involved in the enzymatic reactions which regulate lipid transport. In a long-term (20 weeks) experiment (table 10) the terminal determination of lipids and their fractions in the serum and liver showed in the groups of rats receiving B_{12} the reverse of the findings in the control group and in the groups receiving supplements of choline and methionine, given separately.

Among the substances involved in the reactions of transmethylation, dimethylethanolamine prevented the decrease in serum cholesterol. Unexpectedly homocystine, especially when given in combination with choline and vitamin B_{12}, led to significant hypercholesterolemia. In contrast, cystine had no effect on serum cholesterol whether it was given alone or with lipotropic factors. This difference is remarkable when it is considered that both promote fatty infiltration and fibrosis of the liver (16).[10] The underlying biochemical reactions of hypercholesterolemia produced by homocystine with the common thromboembolic phenomena observed in metabolic homocystinuria (26) is not borne out by findings of low (27) or low normal [11] cholesterol levels in homocystinuria. The observations with homocystine and cystine in the present experiments are not in accord with findings of hypercholesterolemia in rats fed a diet low in sulfur-containing amino acids, corrected by addition of these acids (28).

Long-term studies in the literature on the relation of fatty acids in the body to the composition of the diet have been limited to the use of normal rations or of rations low in protein but containing sufficient amounts of lipotropic factors to prevent dietary hepatic injury (29–32). The results may be summarized in the statement that progressive approximation in the composition of liver fat and adipose tissue to that of the fats used was the rule. Our own long-term experiment showed differences in the composition of the fatty acids in the liver, serum and adipose tissue, depending on the absence or presence of lipotropic factors.

As with the other parameters of lipid metabolism, the group which stood out as different from the rest was that receiving the supplement of vitamin B_{12}. In the liver, arachidonic acid was present in high proportion. Oleic and high unsaturated fatty acids were lower than in the other groups. In the serum, oleic as well as arachidonic acid was high, even in relation to total lipids. The serum fatty acids of the choline-treated group and, to a much less extent, of the animals supplemented with methionine, approached in relative proportion, the values of the vitamin B_{12} group. The fatty acids in the liver of the groups supplemented with choline and methionine showed, in several instances analogous values, different from the results in either control or vitamin B_{12}-supplemented groups (for example, a lower arachidonic

[10] See tables 7 and 8 (16).
[11] Barness, L. A., Hospital of the University of Pennsylvania, personal communication.

acid and higher linoleic acid than that of the controls). The depot (perirenal) fatty acids in all groups had a composition gradually approaching that of the dietary fat. Linoleic was higher in the vitamin B_{12}- and choline-supplemented groups than in the controls or the group receiving methionine. Highly unsaturated fatty acids characteristic for cod liver oil, present in the diet, were absent in the perirenal depot fat.

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Effect of Protein Level on Utilization of Casein and Gluten by Weanling Rats [1]

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ABSTRACT The effect of protein level on utilization of casein and gluten by weanling rats was studied over a wide range of concentrations, from 5.1 to 59.4 protein calories per cent total calories (P%) for casein, and from 6.6 to 58.5 for gluten. The decrease in net protein utilization (NPU) with the increase in protein concentration follows a semilogarithmic curve; NPU casein equals $-56.54 \log P + 121.92$ and NPU gluten equals $-24.35 \log P + 57.77$. An equation which permits the prediction of NPU from score and protein concentration is proposed. Protein values as net dietary protein calories % (NDpCals %), calculated using predicted figures for NPU agree well with experimental values. The relationship between protein intake, as net dietary protein calories per $kg^{0.73}$ ($NDpCals/kg^{0.73}$) and weight gain could also permit the calculation of weight increments from predicted protein values.

The recent joint FAO/WHO report on protein requirements (1) uses net dietary protein calories % (NDpCals %) as an expression for protein value of human diets (2). NDpCals % is the percentage of the total calories as wholly utilizable protein, and is the product of protein concentration, as protein calories per cent total calories (P %), by protein quality, measured as net protein utilization (NPU).

Chemical scoring of the protein is indicative of protein quality only at low levels of concentration and cannot always be used as a measure of protein quality. NPU must be biologically determined at the concentration at which the protein occurs in the diet, because protein utilization falls off with the increase in protein-to-calorie ratio (3). If the biological assay is to be avoided, it becomes important to predict protein utilization and protein value from chemical score and protein-to-calorie ratio. Miller and Payne (3) have proposed a general equation for predicting NDpCals % of diets from chemical score at any protein concentration.

We have studied the effect of protein-to-calorie ratio on the net protein utilization of casein and gluten diets, and arrived at a simple equation that predicts well the protein value of these diets, but differs substantially from that proposed by Miller and Payne.

PROCEDURES [1]

The determination of protein quality as net protein utilization was carried out in duplicate according to the method of Miller and Bender (4) in a 10-day feeding trial, using 31-day-old albino rats of our stock.

Casein and gluten diets [2] with increasing quantities of protein were prepared according to the general formula: (in grams) protein, varied; cornstarch, (500 minus protein); vegetable fat, 150; and vitamin and mineral mixture (6), 350. For each diet the increase in protein was compensated for by a decrease in carbohydrate. The calorie values of the diets were determined from chemical analysis using Atwater's factors and the protein calories per cent total calories (P %) for gluten and casein diets was calculated according to Miller and Payne (3).

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[1] The abbreviations used are: P, protein-to-calorie ratio; P %, a ratio of protein calories to per cent total calories; *experimental NPU*, net protein utilization determined experimentally (4); *predicted NPU*, calculated according to the formula we propose; *S*, protein score derived from amino acid data (5); *NDpCals %*, (net dietary protein calories %), percentage of the total calories of the diet supplied as wholly utilizable protein; *experimental NDpCals %*, (experimental NPU × P) × 100; *predicted NDpCals %*, (predicted NPU × P)/100; *calorie intake*, diet consumed in kilocalories; *NDpCals intake*, (calorie intake × NDpCals %)/100.

[2] Obtained from Nutritional Biochemicals Corporation, Cleveland.

RESULTS AND DISCUSSION

There is general agreement that the utilization of nitrogen from a given protein source decreases with the increase of the protein-to-calorie ratio in the diet (3, 7–10). However, the way in which this decrease occurs is a matter of controversy. Miller and Payne (3) have reported that the relationship between net protein utilization (NPU) and the level of protein fed was always substantially linear and that the slope depends on the nature of the protein. On the other hand, Morrison (8) has given evidence indicating, for 5 different proteins examined, the decrease is more adapted to a semilogarithmic model.

Figure 1 shows the decrease of NPU for casein and gluten with the increase in protein concentration in the diet. It can be seen that the semilogarithmic curve is better-adapted to the experimental results than the linear model of Miller and Payne, especially at high concentrations. These results (NPU casein = − 56.54 logP + 121.92 and NPU gluten = − 24.35 logP + 57.77) are in good agreement with those given by Morrison (8) for gluten and casein.

We have attempted to generalize the decrease in NPU with the increase in protein-to-calorie ratio by an equation that considers: a) maximal protein utilization, equatable with chemical score, which occurs at or below the level of 4 NDpCals % (3), and b) minimal protein utilization at 9 NPU units, when P equals 100%. This value was obtained as the mean of the intercepts (gluten 9.1, casein 8.8) in the ordinate of the logarithmic relationships shown in figure 2.

In this way we have arrived at the following equation:

$$NPU = 9 + \frac{(9-S)(\log P - 2)}{\log S - 0.6}.$$

At values of $S \times P \leq 400$, that is, equal to or lower than the protein level sufficient to maintain nitrogen equilibrium (3), protein utilization attains its maximum and NPU equals S.

The equation we propose has been derived from the study of 2 proteins that differ widely in quality (scores 80 and 40 (5)); and could be applied to any protein whose score fits in this range.

Figure 2 shows the relationship between the experimental values and the proposed equation. From the predicted NPU, the net dietary protein calories % (NDpCals %) of the different diets were calculated. These are shown in table 1 together with the experimental results and those calculated according to the prediction of Miller and Payne. Eight out of fourteen predicted values for casein and ten out of twelve for gluten,

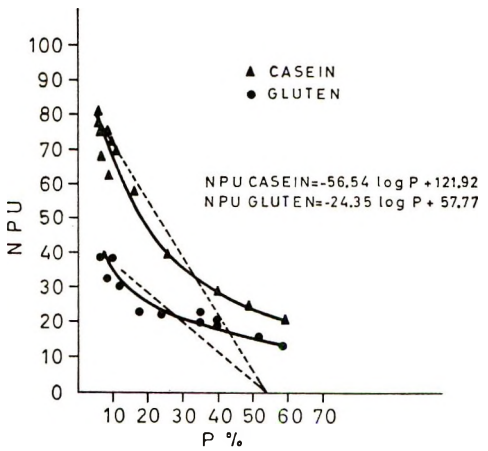

Fig. 1 Net protein utilization of casein and gluten at different protein-to-calorie ratios. The curves represent regression lines of best fit for all experimental values. The straight lines were drawn according to the equation of Miller and Payne (3) relating NPU to protein concentration.

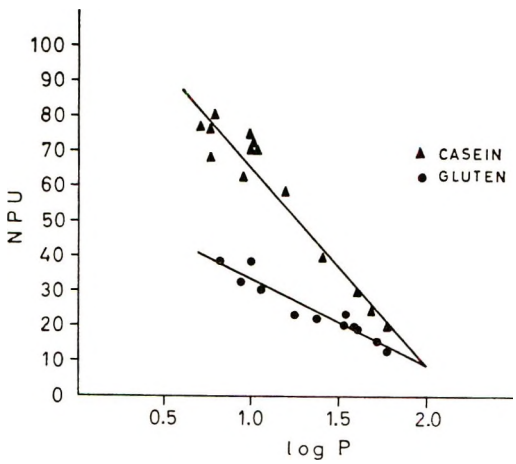

Fig. 2 Net protein utilization of casein and gluten at different protein-to-calorie ratios. The lines were drawn according to the equation proposed in the text, using 80 as the score for casein and 40 for gluten.

TABLE 1

Weight gain and NDpCals intake [1] of weaning rats fed casein and gluten diets of increasing protein-to-calorie ratio (10-day period); comparison of experimental and predicted protein value

Protein-to-calorie ratio	Net protein utilization (NPU)	Experimental NDpCals % [2]	Predicted NDpCals % (Tagle and Donoso) [3]	Predicted NDpCals % (Miller and Payne (3))	NDpCals [4] / $kg^{0.73}$	Change in body wt (experimental)
						g
			Casein diets			
5.1	76.7	3.9	4.1	4.1	83	3.5
5.8	68.1	4.0	4.4	4.6	80	3.1
5.8	76.0	4.4	4.4	4.6	80	4.2
6.1	80.5	4.9	4.6	4.7	80	3.2
8.9	62.2	5.5	5.9	6.5	108	8.2
9.9	74.0	7.3	6.3	7.2	161	26.3
10.0	71.9	7.2	6.4	7.2	146	20.0
10.5	70.7	7.4	6.6	7.4	145	17.2
10.7	70.0	7.5	6.6	7.5	175	28.8
15.3	57.9	8.9	8.2	9.5	190	38.8
25.5	39.3	10.0	10.6	11.8	224	43.3
39.6	29.5	11.7	12.3	9.2	241	42.9
48.6	24.4	11.9	12.7	4.2	254	43.3
59.4	20.0	11.9	12.7	—	243	41.3
			Gluten diets			
6.6	38.5	2.6	3.0	2.6	49	−3.6
8.7	32.7	2.8	3.6	3.5	52	−2.8
9.8	38.8	3.9	3.9	3.9	68	−0.7
11.2	30.9	3.5	4.3	4.3	71	1.9
17.5	23.3	4.1	5.7	5.7	96	7.7
23.4	22.4	5.3	6.0	6.4	111	11.2
34.1	20.9	7.1	8.0	6.1	152	19.4
34.8	23.7	8.3	8.1	6.1	162	20.7
39.2	19.8	7.7	8.5	5.3	176	23.4
39.9	19.5	7.8	8.5	5.1	166	23.8
51.7	16.2	8.4	9.3	1.1	132	13.9
58.5	13.0	7.5	9.5	—	117	10.7

[1] (Calorie intake multiplied by NDpCals %)/100.
[2] (Experimental NPU × P)/100.
[3] (Predicted NPU × P)/100; calculated as explained in Procedures section.
[4] (Calorie intake × NDpCals %)/100.

agree as well with the experimental values, or better than those calculated with the Miller and Payne equation. Our prediction is more accurate than theirs at higher levels of protein intake (table 1); when $P > 54\%$ we can predict and their equation does not.

Figure 3 shows the experimental and predicted protein values of the different diets. The overall agreement can be considered good, as judged by a correlation coefficient of 0.96.

According to Miller and Payne (3) the diets with more than 50% protein calories do not support growth in the rat. This is clearly not the case with our rats, as can be seen from table 1; however we have no explanation for this disagreement.

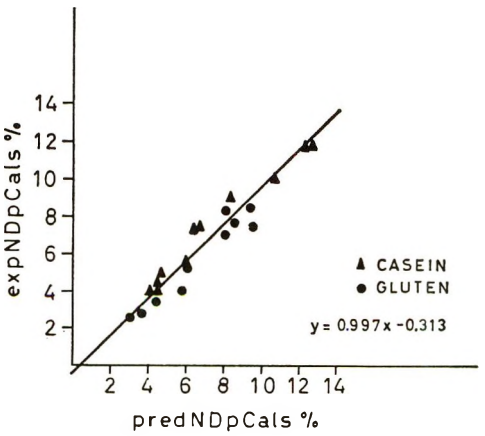

Fig. 3 Net dietary protein calories % of casein and gluten diets. Experimental versus predicted values.

Miller and Payne (7) have shown that the increase in body weight is proportional to the net dietary protein calories intake per $kg^{0.73}$, more so than to the NDpCals % of the diet. Our results agree with this finding (fig. 4). A corollary to this fact appears to be that requirements for growth cannot be stated solely on the protein value of diets (as NDpCals %), some measure of the intake, both protein and calorie, being also necessary. It is of interest that the value for NDpCals/$kg^{0.73}$/day when the change in body weight (g) is zero (fig. 4B) equals 6.8. This figure is in good agreement with that used by Miller and Payne (7) and ourselves (6) when calculating endogenous nitrogen, that is, 6.0 NDpCals/$kg^{0.73}$ per day or 250 mg N/$kg^{0.73}$ per day.

The relationship between NDpCals/$kg^{0.73}$ intake and the weight gain (fig. 4B) could permit the calculation of weight gain from predicted protein values. However, in the prediction of weight increase for other species than the rat and for longer periods than the 10 days considered in the trials used, the influence of growth rate and therefore of the amount of nitrogen used for maintenance, limits the possibility of using this relationship, and exponential equations of the type proposed by Miller and Payne (7) should be used.

The mechanisms through which the decrease in utilization of protein occurs are not known. Miller and Payne (7) have theorized that the protein synthesis (maintenance and growth) requires a fixed amount of energy, and that therefore with the increase in protein concentration less and less energy (non-protein calories) would be available for this purpose.

An explanation at another level can be sought through the adaptative nature of the enzymes of the urea cycle, shown by Schimke (11). This author describes higher activities with increased protein concentrations in the diet. We have compared the levels given by Schimke for arginine succinate synthetase in the liver of the rat fed 15, 30 and 60% casein diets with our prediction for the NPU of his diets and found that the increase in enzyme activity strictly parallels the decrease in NPU. At low levels of protein intake the free amino acids that enter the liver pool would have a smaller possibility of being channeled into the urea cycle than when the concentration of protein in the diet is high. By escaping the catabolic pathway the amino acids would have a larger chance of being incorporated into protein, thereby increasing the utilization of the protein intake.

It can be very well argued that predictions of protein value are important only

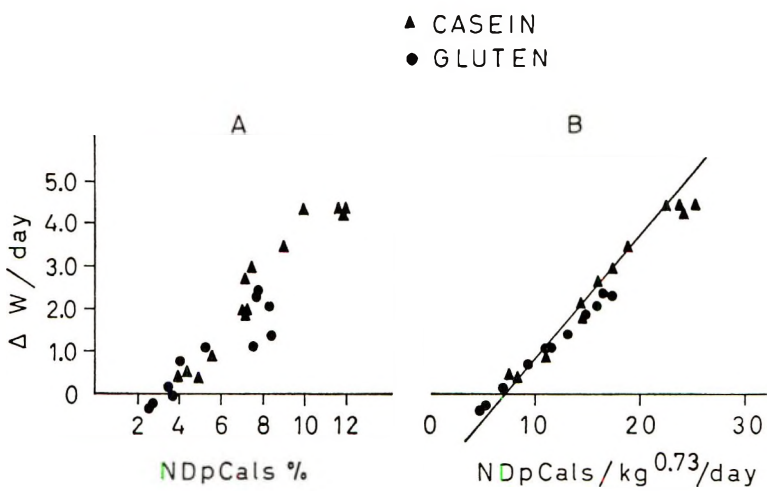

Fig. 4 *A*, Weight gain of weanling rats fed casein and gluten diets of different net dietary protein calories %; *B*, weight gain of the same rats versus their intake expressed in NDpCals/$kg^{0.73}$ per day.

at levels of protein concentration found in practical human diets (that very rarely go beyond 25% protein calories) and at these levels Miller and Payne's prediction is as good as the one proposed. However, the prediction of NDpCals % at high concentrations of protein in the diet can become important when protein-rich foods are used or when a high protein concentration is used instead of good biological quality to obtain adequate protein values.

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letters

AMINO ACID IMBALANCE OR DEFICIENCY

The interesting report of Hartman and King (1) suggests that an amino acid imbalance is a distinct and unique phenomenon. As early as 1960 and 1961 (2, 3) results from our laboratory indicated no impairment in the utilization of the limiting essential amino acid in an "imbalanced" diet, stressing the close identity of "imbalance" with amino acid deficiency. In the same vein, Harper and associates (4) recently concluded that "the limiting amino acid in an imbalanced diet is used as efficiently, if not more efficiently, than the same amino acid in an appropriate control."

The purpose of this communication is to point to the similarity of the results obtained by Hartman and King using an "imbalanced" diet and those of Sidransky et al. (5) working with an uncomplicated threonine deficiency. Under both conditions there was enhanced liver protein synthesis (or amino acid incorporation), thus corroborating earlier suggestions of a close similarity, if not identity, between amino acid imbalance and deficiency.

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THE AUTHOR'S REPLY:

Dr. Fisher's comments are appropriately put, though I am of the opinion that it is still useful to use the term "amino acid imbalance" to describe a particular type of amino acid deficiency, namely, that situation in which a small reduction in the dietary concentration of a limiting essential amino acid results in an inordinate amount of anorexia and consequent growth failure.

Consider, for example, the dietary systems used in the paper by Hartman and me to which Dr. Fisher referred. A fully "corrected" diet contains 0.375% histidine and supports weanling rat growth of 6.2 g/day with a PER of 4.1. Reducing the histidine concentration to 0.25% results in daily gains of only 1.6 g with a PER of 1.7 (Ph.D. dissertation, E. S. Ellison, Virginia Polytechnic Institute, 1967).

A second characteristic of "amino acid imbalance" situations exists when a basal rather than a corrected diet is used as the control. In the system in question, for example, the basal diet contains 10% protein and 0.25% histidine. It supports daily weight gains of 10.1 g at a PER of 3.5. Adding the other essential amino acids to the basal diet to give 15% protein with the

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same 0.25% histidine results in daily gains of 5 g at a PER of 0.5 (Ph.D. dissertation, E. S. Ellison, Virginia Polytechnic Institute, 1967).

The particular deficiency in "imbalanced" diets, then, is one, relative to other amino acids, not an absolute deficiency. Like Harper, we feel there is need for continued use of the term "amino acid imbalance" to distinguish such situations from deficiencies in the absolute concentration of an amino acid.

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