Influence of Altered Thyroid Status on the Food Intake and Growth of Rats Fed a Thiamine-deficient Diet [1,2]

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ABSTRACT Hypothyroidism, produced by the feeding of propylthiouracil, stimulates the food intake and growth of rats fed a thiamine-deficient diet. In contrast, hyperthyroidism, produced by the administration of thyroxine, exacerbates the growth retardation of rats fed a thiamine-deficient diet without affecting the food intake. Since thiamine deficiency per se causes mild thyroid atrophy, it is postulated that thyroid function is an integral part of a feedback mechanism controlling food intake in thiamine deficiency.

The relationship between thiamine need and thyroid status has been the subject of many investigations (reviews: 1, 2). Much of the early interest in this relationship stemmed from the increase in the requirement for thiamine observed in the hyperthyroid dog (3, 4), pigeon (5) and rat (6–9). This increase in requirement has been adequately explained on the basis of the overall acceleration of metabolic processes brought about by thyroxine (10, 11).

In the process of studying the increased need for thiamine during hyperthyroidism, however, investigators have overlooked other interesting relationships between thyroid status and thiamine. In particular, little attention has been given to the effect of hyperthyroidism on shortening the time required for onset of the anorexia which first characterizes deficiency (3, 4). Investigation of this relationship could provide information on the appetitive mechanisms which fail in the presence of a dietary deficiency of thiamine.

The apparent connection between thyroid status and food intake behavior has been based largely on the effect of the calorigenic response to cold on appetite. Rats exposed to a cold environment or rendered hyperthyroidic by daily injections of thyroxine increase their food intake (12, 13). In addition, a portion of the appetite of the rat is unaffected by a thiamine deficiency and is stimulated in the cold (14). Although small doses of thyroxine fail to stimulate the food intake of rats kept at room temperature (15), additional study in this area appeared warranted.

We have investigated the effect of altered thyroid function on the food intake behavior of rats fed a thiamine-deficient diet. The effects of hypothyroidism and hyperthyroidism are reported.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain, initially weighing 70 to 100 g, were housed in individual steel cages with wire-mesh bottoms in a temperature- and humidity-controlled room with a constant light-dark cycle of 12 hours. Fresh water and experimental diets (table 1) were provided ad libitum. A minimal amount of thiamine (0.4 μg thiamine/g) was included in the thiamine-deficient diet to produce a controlled rate of onset of the deficiency. Food intakes were recorded daily and the rats were weighed three times a week. There were five animals in each experimental group.

Hypothyroidism was produced by feeding experimental diets modified to contain 0.01, 0.05 and 0.10% 6-propyl-2-thiouracil (PTU). A mixture of PTU and sucrose (100 mg PTU/g sucrose) was added to the

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TABLE 1

Experimental diets

Ingredient	Control [1]	Deficient [2]
	%	%
Casein	22.0	22.0
Sucrose	17.1	17.5
Dextrose	30.0	30.0
Dextrin	15.0	15.0
Salt mix W [3]	4.1	4.1
Vitamin mix [4]	1.0	1.0
Choline chloride [5]	0.2	0.2
Corn oil	10.0	10.0
Thiamine mix [6]	0.6	0.2

[1] Diet contained 1.2 μg thiamine/g.
[2] Diet contained 0.4 μg thiamine/g.
[3] Wesson, L. G. 1932 A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Science, 75: 339.
[4] The vitamin mix provided the following per 100 g of diet: (in International Units) vitamin A, 400; vitamin D, 40; and vitamin E, 25; (in milligrams) vitamin K, 0.05; riboflavin, 0.5; niacin, 2.5; pyridoxine, 0.5; calcium pantothenate, 2.0; folic acid, 0.5; biotin, 0.02; and vitamin B_{12}, 0.003.
[5] Choline chloride–water (1:1).
[6] Two hundred micrograms thiamine·HCl per gram ball-milled sucrose. Obtained from Calbiochem, Los Angeles, Calif.

diet at the expense of sucrose. According to Suzuki and O'Neal (16), a level of 0.01% PTU in the diet may be regarded as a therapeutic dose because it produces a satisfactory degree of hypothyroidism in the rat without affecting the general physical condition of the animal. A level of 0.10% PTU in the diet, however, is considered toxic.

Hyperthyroidism was produced by the subcutaneous injection of graded levels of thyroxine. The rats were injected with 10, 25, 50 or 100 μg thyroxine every 4 days. The thyroxine was suspended in 0.9% saline to provide 100 μg thyroxine/ml, and appropriate amounts were injected. Because the dose of thyroxine considered physiologic for the rat is 25 to 35 μg every 4 days (17) only the 100-μg level should be considered thyrotoxic in this study. The effect of hyperthyroidism on the histology of the thyroid gland was also determined.

RESULTS

Hypothyroidism affected the food intake and growth of rats fed the control and thiamine-deficient diets. Feeding PTU depressed the growth of rats fed the control diet (fig. 1a). The therapeutic level (0.01% PTU) depressed growth after 12 days whereas the toxic level (0.10% PTU) depressed growth from the start.

In contrast, feeding PTU protected against the growth depression caused by thiamine deficiency (fig. 1 b). The therapeutic level afforded the best protection but even the toxic level, which initially depressed growth, afforded some protection against weight loss.

Both therapeutic and toxic levels of PTU in the control diet depressed food intake (table 2). The more severe depression at the 0.10% level reflects the greater growth depression. In contrast, the therapeutic level of PTU (0.01%) in the thiamine-

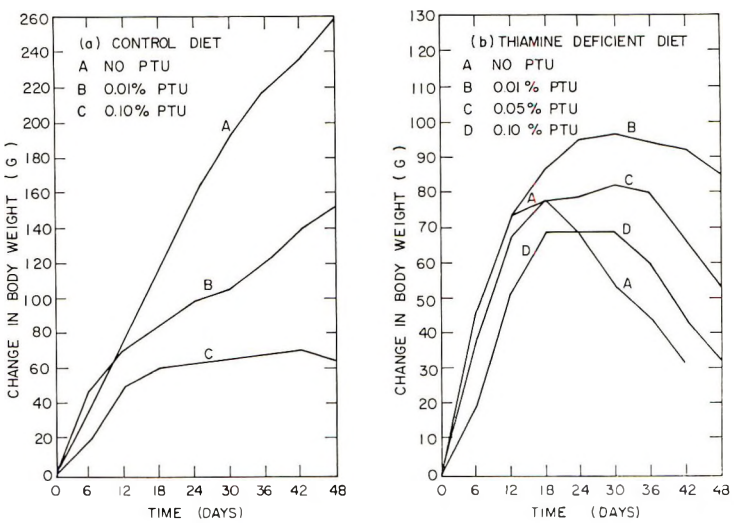

Fig. 1 Effect of propylthiouracil (PTU) on growth of rats.

TABLE 2

Effect of propylthiouracil (PTU) on the cumulative food intake (g) of rats [1,2]

Day	% PTU in diet			
	0	0.01	0.05	0.10
		Control diet		
7	94.0 ± 7.8	106.4 ± 6.6		88.1 ± 12.9
14	211.1 ± 13.3	207.9 ± 8.3		182.1 ± 25.6
21	346.6 ± 17.9	299.5 ± 15.7		261.2 ± 32.8
28	476.7 ± 16.2	380.7 ± 22.3		323.3 ± 39.0
35	612.9 ± 23.7	453.8 ± 26.3		384.3 ± 46.2
42	735.7 ± 25.5	541.9 ± 34.1		440.8 ± 50.8
49	853.0 ± 19.9	622.3 ± 45.2		489.4 ± 98.7
		Thiamine-deficient diet		
7	107.8 ± 2.6	107.9 ± 4.9	97.2 ± 8.9	81.4 ± 4.8
14	209.9 ± 12.6	214.2 ± 10.3	194.9 ± 18.1	178.9 ± 5.0
21	287.7 ± 24.5	305.6 ± 14.4	273.0 ± 19.8	259.0 ± 6.1
28	332.7 ± 21.5	377.5 ± 19.0	334.1 ± 21.5	323.5 ± 17.1
35	374.0 ± 19.8	435.6 ± 28.1	390.7 ± 23.4	375.1 ± 17.3
42	407.0 ± 21.8	489.0 ± 31.2	426.2 ± 24.2	410.9 ± 17.8
49	448.6 ± 26.7 [3]	536.7 ± 36.2	454.0 ± 24.7	442.2 ± 21.1

[1] Each value represents the mean ± SD.
[2] There were five rats in each group.
[3] Four rats were used to obtain this value.

TABLE 3

Statistical analysis of the effect on cumulative food intake of thiamine and propylthiouracil in the diet [1]

Source of variation	F value				
	Day 14	Day 21	Day 28	Day 35	Day 42
Thiamine	0.26	10.11 **	68.10 **	135.86 **	207.77 **
Propylthiouracil	0.12	3.09	8.29 *	19.14 **	18.42 **
Interaction	0.55	15.32 **	62.6 **	100.12 **	107.95 **

[1] The analysis of variance was done on data obtained from the control, control + 0.01% PTU, thiamine deficient and thiamine deficient + 0.01% PTU groups.
* Significant at the 5% level.
** Significant at the 1% level.

deficient diet stimulated food intake. The higher levels of PTU, however, did not stimulate intake of the deficient diet.

Statistical treatment of the food intake data (table 3) indicates that the thiamine content of the diet exerted a highly significant effect on food intake as early as day 21. The level of PTU in the diet did not exert a similar effect on food intake until day 35. More importantly, a very significant interaction between thiamine level and PTU in the diet was found after day 21. These results indicate that hypothyroidism protected against the anorexia due to thiamine deficiency.

Hyperthyroidism had little effect on the growth of rats fed the control diet (fig. 2). However, it exacerbated the weight loss of rats fed the thiamine-deficient diet. The greater the dose of thyroxine, the more rapid and severe the effect on body weight loss and the more rapid the appearance of polyneuritis. In addition, the first death in each thiamine-deficient group was directly related to the level of thyroxine (table 4).

TABLE 4

Day of first death in groups of thiamine-deficient animals administered graded doses of thyroxine

Thyroxine dose	Day of first death
μg/4 days	
0	38
25	34
50	25
100	22

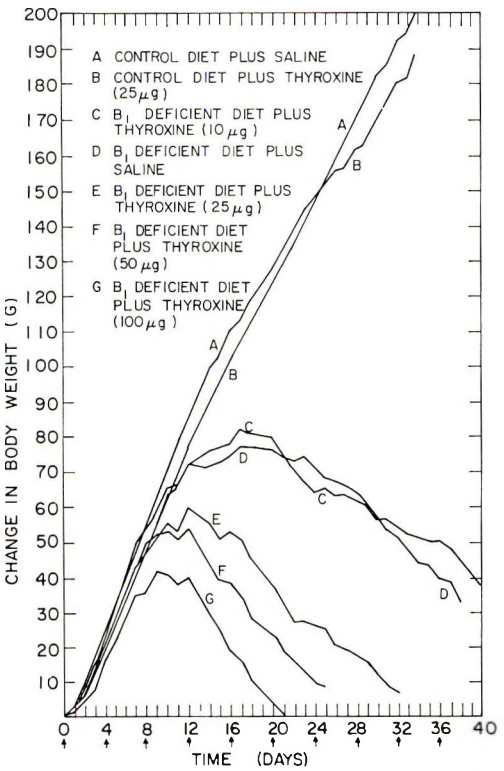

Fig. 2 Growth rate of rats injected with graded doses of thyroxine every 4 days.

Hyperthyroidism failed to affect the food intake of rats fed either the control or thiamine-deficient diet (table 5). Although a slight increase (9.1 g) in intake of the deficient diet by thyroxine-treated rats was observed after 14 days, the difference is not statistically significant (Student's *t* test). The 28-day food intakes of the thyroxine- and nonthyroxine-treated rats fed the thiamine-deficient diet were remarkably similar. This is significant in view of the marked difference in body weights between the thyroxine- and nonthyroxine-treated animals (fig. 2).

After 3 weeks hyperthyroidism caused a marked atrophy of the thyroid in rats fed the control diet (fig. 3) but only a moderate atrophy in rats fed the thiamine-deficient diet (fig. 4). Of particular interest is the fact that thiamine deficiency itself resulted in a mild atrophy of the thyroid (fig. 4). No changes were observed in the thyroids of control rats pair-fed to the thiamine-deficient animals.

DISCUSSION

Interpretation of much of the early work on the relationship of thyroid status to thiamine need is difficult because of the variety of experimental techniques employed. Primarily, the degree of hyperthyroidism produced by the feeding of desiccated thyroid (3, 5, 18–21), the feeding of thyroxine (22), or the injection of milligram amounts of thyroxine (9, 23) is not known. In contrast, the degree of alteration in thyroid status in the present study may be considered to be in the physiologic rather than the toxic range.

Tata and co-workers (17) reported that chronic hyperthyroidism can be produced in the rat by subcutaneous injection of 25 to 35 μg thyroxine every 4 days. The basal metabolic rate was increased 45% and could be maintained at this level for 2 to 3 months without any visible signs of toxicity or any interference with growth. Evidence of the physiologic nature of the doses of thyroxine administered in our study is provided by the histologic changes produced in the thyroid glands of rats fed the con-

TABLE 5

Effect of thyroxine on the cumulative food intake (g) of rats fed the control and thiamine-deficient diets [1,2]

Day	Control	Control + thyroxine	Thiamine deficient	Thiamine deficient + thyroxine
8	105.3 ± 2.2	104.5 ± 13.8	95.1 ± 6.1	105.6 ± 7.8
14	195.1 ± 11.1	196.5 ± 24.3	173.3 ± 12.1	182.4 ± 16.9
16	223.4 ± 12.2	229.7 ± 26.0	194.9 ± 13.0	199.3 ± 18.5
18	253.6 ± 12.9	265.8 ± 26.6	215.2 ± 14.0	212.5 ± 19.2
24	346.7 ± 22.6	374.3 ± 39.4	255.3 ± 11.4	248.4 ± 24.0
28	418.5 ± 26.8	445.3 ± 48.8	276.9 ± 16.3	270.0 ± 27.3

[1] The level of thyroxine intake was 50 μg every 4 days.
[2] Each value represents the mean ± SD.

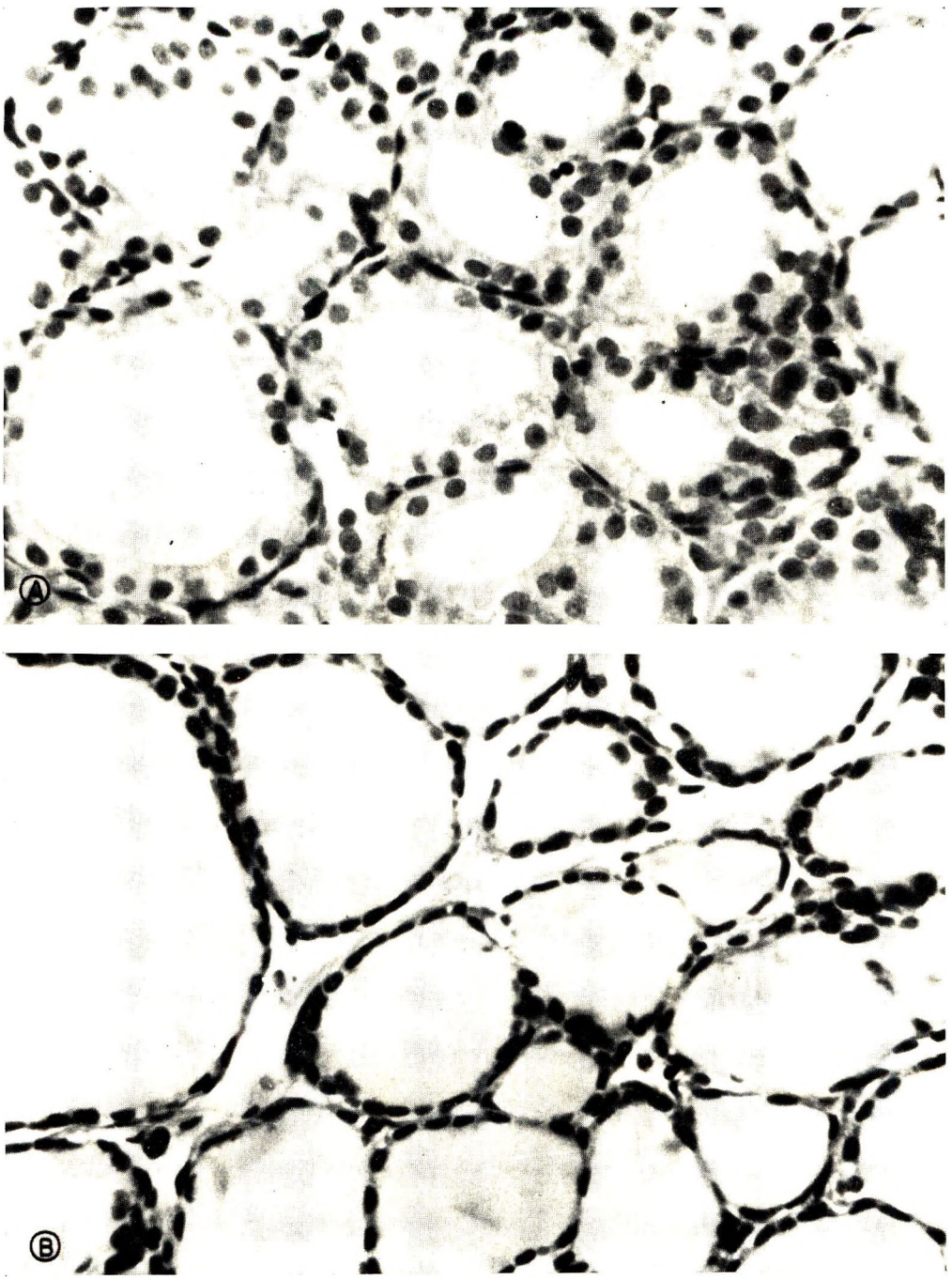

Fig. 3 Thyroid of rats fed the control diet. A: no thyroxine. B: thyroxine.

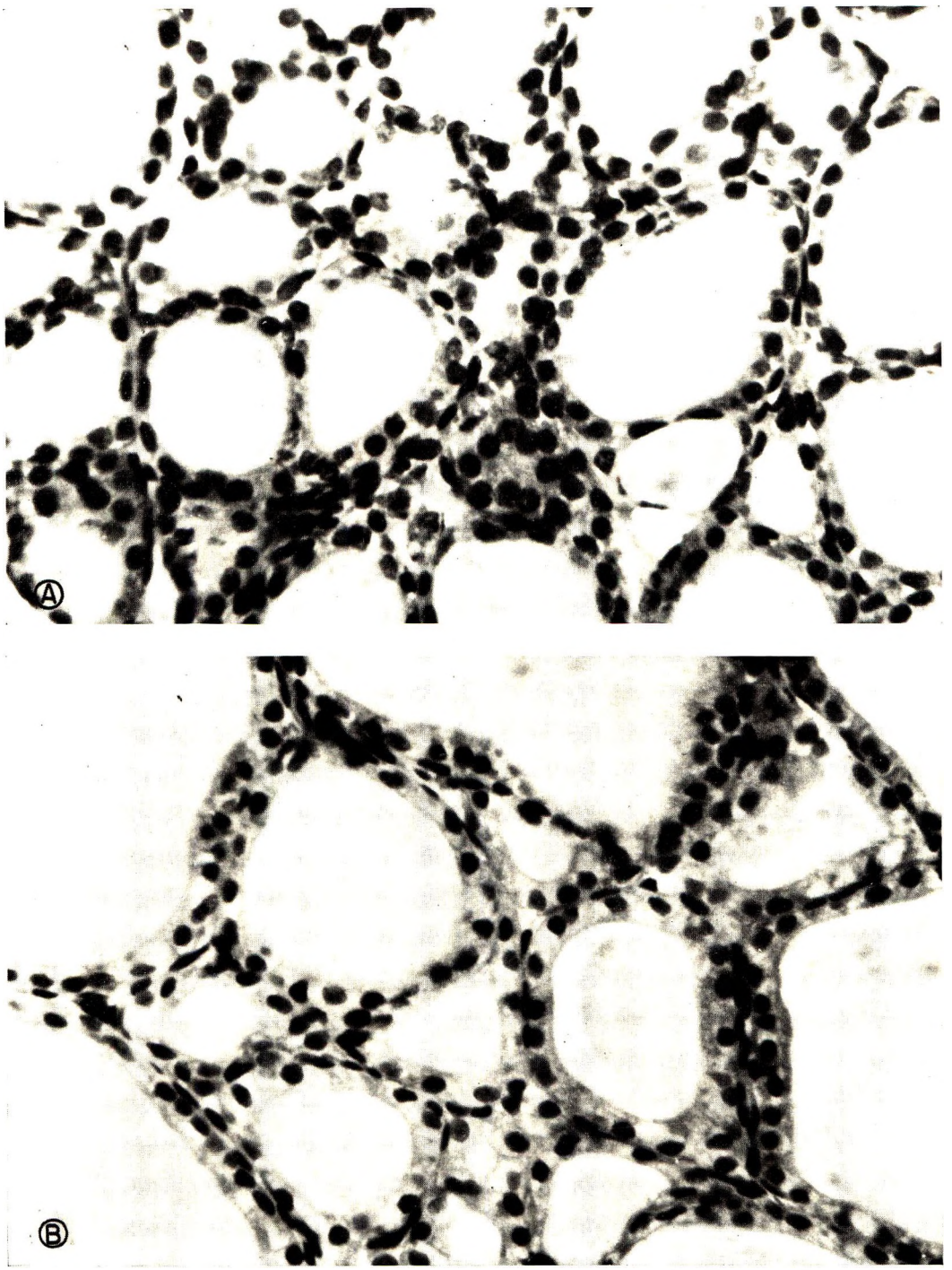

Fig. 4 Thyroid of rats fed the thiamine-deficient diet. A: no thyroxine. B: thyroxine.

trol diet (fig. 3). Although marked atrophy of the thyroid occurred, the rats still showed a normal growth rate (fig. 2). Atrophy of the thyroid is the result of increased circulating thyroxine which causes a reduction in thyrotropin (thyroid-stimulating hormone) release from the pituitary gland (24). Since the atrophic thyroid fails to release thyroxine, the exogenous thyroxine is responsible for maintenance of the normal growth pattern and the elevated metabolic rate.

Similarly, the degree of hypothyroidism produced in the rat by feeding propylthiouracil (PTU) at the 0.01% level in the diet may be regarded as therapeutic or physiologic. PTU, at the 0.01% level, produced a satisfactory degree of hypothyroidism in the rat without affecting the general physical condition of the animal (16).

The feeding of PTU, which blocks thyroid function, protected the rat from thiamine deficiency and increased survival time (fig. 1). It is tempting to speculate that the thyroid atrophy found in the non-thyroxine-treated thiamine-deficient rats (fig. 4) indicates an attempt by the animal to defend itself against the deficiency. The rat could increase survival time by reducing thyroid function. Administration of exogenous thyroxine, however, negates this attempt, as evidenced by the more rapid demise of the thyroxine-treated animals (fig. 2).

Since thyroid atrophy was not found in pair-fed control rats, the effect of a diminished food intake on this histologic change can be ruled out. This finding is significant since many of the histologic changes in thiamine-deficient rats are due to a reduced food intake (25). The thyroid atrophy observed was not completely unexpected, however, since Drill (1), reviewing the effects of thiamine deficiency on the thyroid gland, reported some instances of atrophy. Similarly, Blaizot and Blaizot (2) reported considerable alteration of the colloid in the thyroid of rats fed thiamine-deficient diets.

The protection against thiamine deficiency afforded by hypothyroidism is, in some respects, similar to the sparing action of dietary fat on the thiamine requirement (26, 27). In both instances anorexia is delayed and survival time is increased. The sparing action of dietary fat can be explained on a biochemical basis as less need for thiamine since fatty acids can be converted to acetate without the participation of the thiamine-requiring pyruvic dehydrogenase system. The lowered need for thiamine in hypothyroidism, however, is probably due to the decrease in metabolic rate in much the same manner as the increased thiamine need in hyperthyroidism is due to the increase in metabolic rate (10).

The ability of the hypothyroid rat fed the thiamine-deficient diet to delay anorexia (table 2) indicated that this anorexia was related more to the thiamine content of the tissues and the demands of metabolism than to the thiamine content of the diet. As long as there is sufficient tissue thiamine to participate in metabolic reactions, the rat will continue to eat a thiamine-deficient diet. The lowered metabolic rate in hypothyroidism spares tissue thiamine and thus delays anorexia.

The apparent relationship between thiamine deficiency and reduced thyroid function is further enhanced by the finding that thiamine deprivation has a marked effect on rectal temperature in rats (28). Since oxidative mechanisms and, therefore, heat production are influenced by thyroid activity, any interference with thyroid function may affect body temperature. Veen et al. (28) pointed out that if triiodothyroacetic acid was the active thyroid principle, the conversion of triiodothyropyruvate to the acetate would proceed by the thiamine-requiring pyruvic dehydrogenase system. Thus, a thiamine deficiency could block thyroid function and consequently reduce oxidative metabolism and energy production by reducing the formation of triiodothyroacetic acid.

The failure of thyroxine to enhance food intake (table 5) indicated that the thyroxine elicited in the calorigenic response to cold is not directly responsible for the increase in intake of the thiamine-deficient diets by rats kept at 5° (14). The results of our study concur with the finding of Groisse and Turner (15) that low doses of thyroxine do not stimulate the food intake of rats, but differ from the finding of Hsieh and Ti (13) that thyroxine causes an increase in food intake in the rat. This

discrepancy is probably related to the difference in amounts of thyroxine tested.

Blaxter (29) explained the increased appetite for thiamine-deficient diets in the cold on the basis that metabolic processes of dissimilation (conversion of energy to heat), characteristic of the calorigenic response to cold, may not require as much thiamine as processes of synthesis. In contrast, our observation that hyperthyroidism exacerbated thiamine deficiency indicates a more rapid utilization of thiamine at a time when the rats were losing weight and expending large amounts of energy. In some respects this finding is similar to the increased need for thiamine in the cold attributed to the demands of a stimulated metabolic rate.

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Zinc Deficiency and Oxidation of L-Methionine-methyl-^{14}C in Rats

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ABSTRACT Studies were undertaken to determine the effect of zinc deficiency on the patterns of oxidation of various ^{14}C-labeled compounds to respiratory CO_2. Significantly higher percentages of L-methionine-methyl-^{14}C were oxidized by zinc-deficient rats. This increase was apparently not due to differences in body weight and was observed with tracer as well as with carrier doses of the amino acid. Intraperitoneal injection of 400 μg zinc daily for 3 consecutive days prevented this occurrence. The specific involvement of the methyl group of methionine was indicated by the observation of normal oxidations of methionine-1-^{14}C and methionine-2-^{14}C. Oxidations of tracer doses of ^{14}C-labeled acetate, glutamic acid and formic acid were essentially unaffected by zinc deficiency. Oxidations of glycine-1-^{14}C, cystine-1-^{14}C, tryptophan-2-^{14}C and formaldehyde-^{14}C, however, were enhanced in zinc-deficient rats.

Previously we reported that zinc deficiency in rats caused a decrease in the activity of pancreatic carboxypeptidase A, but had no effect on pancreatic carboxypeptidase B or liver alcohol dehydrogenase (1). Later Mills et al. (2) found that the reduction of pancreatic carboxypeptidase activity in zinc-deficient rats was abolished on zinc repletion. No work has been done, however, to determine whether these enzymic changes are due to an inadequate supply of zinc for activation or to an impairment in the synthesis of the apoenzyme itself. Because methionine comprises only a small fraction of the rat insulin molecule (3), but is in high concentration in the exocrine enzymes of the pancreas, this amino acid was chosen to determine the role of zinc in pancreatic protein metabolism. During this study, we observed a marked increase in the oxidation of L-methionine-methyl-^{14}C by zinc-deficient rats. Details of these findings are presented here.

EXPERIMENTAL

Animals and diets. Twenty-one to twenty-three-day-old weanling male rats of the McCollum strain obtained from our own colony were generally used for this investigation. In a few experiments, a number of 3-week-old male rats were purchased commercially.[1] At all times, rats of the same origin were randomly divided into two groups according to their age and body weight. Group 1 was kept on a dried egg albumin-sucrose diet with adequate amounts of all required vitamins and minerals except zinc; its composition is shown in table 1. The zinc content of the diet was 2 to 3 ppm as determined by a direct reading spectrometer.[2] Group 2 was used as control rats and received the same diet with a supplementation of 88 ppm zinc as zinc carbonate. All rats were housed in individual stainless steel cages. Feed and deionized water were offered ad libitum.

Zinc determination. Various tissues were excised, cleaned, weighed and ashed in porcelain crucibles at 460 ± 10° after drying at 100°. The residue was dissolved in 1 N HCl and stored in EDTA-washed tubes. The zinc content in each sample was measured with an atomic absorption spectrophotometer using a Boling burner.[3]

Collection and assay of expiratory $^{14}CO_2$. After 14 to 22 days of feeding and an overnight fast, each rat was injected intramus-

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[1] Sprague-Dawley rats were purchased from Zivic Miller Laboratories, Inc., 3848 Hieber Road, Allison Park, Penna. 15101.

[2] Jarrell-Ash Company, Waltham, Mass.

[3] Perkin-Elmer 303, Perkin-Elmer Corporation, Norwalk, Conn.

TABLE 1

Composition of zinc-deficient diet

Ingredients	g/100 g diet
Sucrose	65.97
Dried egg white	15.00
Casein hydrolysate (salt-free) [1]	3.00
Mazola oil [2]	10.00
Salt mixture [3]	5.74
Vitamin supplement [4]	0.29

[1] Acid-hydrolyzed, salt-free, General Biochemicals Inc., Chagrin Falls, Ohio.

[2] Corn Products Company, Argo, Ill.

[3] Furnished per 100 g of diet: (in grams) $CaHPO_4$, 2.716; $CaCO_3$, 0.957; Na_2HPO_4, 0.670; NaCl, 0.383; KCl, 0.670; $MgSO_4$, 0.287; $FeC_6H_5O_7 \cdot 5H_2O$, 0.019; $MnSO_4$, 0.021; KIO_3, 0.001; $ZnCO_3$,* 0.012; and $CuSO_4 \cdot 5H_2O$, 0.001. (* To prepare low zinc diet, $ZnCO_3$ was eliminated from the mixture.)

[4] Furnished per 100 g of diet: (in milligrams) thiamine·HCl, 0.24; riboflavin, 0.60; pyridoxine·HCl, 0.30; Ca pantothenate, 2.00; niacin, 4.00; inositol, 10.00; biotin, 0.20; folic acid, 0.20; vitamin B_{12}, 0.002; choline chloride, 100.00; 2-methyl-1-4-naphthoquinone, 1.0; and α-tocopherol, 6.0; (in IU) vitamin A, 2500; and vitamin D_3, 300.

cularly with ^{14}C-labeled compounds [4] and placed immediately in an individual glass metabolism cage for 1 to 6 hours. Carbon dioxide-free air was supplied by drawing air through a column filled with Drierite and Ascarite with a vacuum pump. The expired $^{14}CO_2$ was collected in a Pyrex conical tube containing the trapping agent: ethanolamine-ethylene glycol monomethyl ether (1:2, v/v). At the end of each collection period, an aliquot of the trapping agent was pipetted into a counting vial and mixed with the scintillation medium of Jeffay and Alvarez (4). The radioactivity was determined in a liquid scintillation spectrometer.[5] Counting efficiency for radiocarbon was about 70%. The ^{14}C standard prepared was the same as that used for intramuscular injection. The dose administered was about 5 μCi/100 g body weight. The exact quantity of solution injected was determined by weighing the syringe before and after. The results obtained from averages of duplicate samples were expressed as percentages of administered ^{14}C recovered as $^{14}CO_2$, at stated time intervals. The calculations of percentage dose recovered do not include quench correction for standard and CO_2 samples since an essentially constant correction factor was obtained from pilot experiments.

RESULTS

The results given in table 2 reveal growth retardation in the rats on a low zinc diet. A majority of the zinc-deficient animals also showed such external symptoms as alopecia, graying of black hair and scabby skin lesions. In addition, the zinc concentrations in the pancreas and kidneys, but not in the liver and testes, of zinc-deficient rats were significantly lower than those of rats fed zinc.

The cumulative, hourly production of $^{14}CO_2$ after L-methionine-methyl-^{14}C injection in zinc-supplemented and zinc-deficient rats was measured (fig. 1). The data show that zinc deficiency resulted in a greatly increased conversion of methionine to CO_2 1 hour after isotope administration. Further increases were noted with respect to the $^{14}CO_2$ collection time. At the end of 4 hours the recovery of injected ^{14}C

[4] The following radiochemicals were obtained from New England Nuclear Corporation, Boston, Mass.: sodium acetate-2-^{14}C (8.56 mCi/mmole); DL-glutamic acid-1-^{14}C (1.97 mCi/mmole); glycine-1-^{14}C (22.3 mCi/mmole); L-leucine-1-^{14}C (34.1 mCi/mmole); L-leucine-UL-^{14}C (240 mCi/mmole); DL-cystine-1-^{14}C (5.7 mCi/mmole); L-methionine-methyl-^{14}C (14.7 mCi/mmole); DL-methionine-2-^{14}C (2.33 mCi/mmole); DL-methionine-1-^{14}C (4.15 mCi/mmole); formaldehyde-^{14}C (12.0 mCi/mmole); and sodium formate-^{14}C (58.7 mCi/mmole). DL-Tryptophan-2-^{14}C (ring labeled) (21 mCi/mmole) was obtained from Schwarz Bio-Research, Inc., Orangeburg, N. Y.

[5] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.

TABLE 2

Growth and zinc contents in selected tissues of zinc-supplemented and zinc-deficient rats

Type of diet	Initial body wt	Final body wt, 16 days	No. of rats	Zinc content			
				Liver	Pancreas	Kidney	Testes
	g	g		μg/g wet wt	μg/g wet wt	μg/g wet wt	μg/g wet wt
Zinc supplemented	48 ± 2 [1]	105 ± 3	7	37.1 ± 5.58	38.8 ± 7.84	28.2 ± 1.38	22.1 ± 1.02
Zinc deficient	49 ± 3	63 ± 5	6	32.2 ± 6.17	22.2 ± 5.68	21.6 ± 2.05	21.9 ± 1.77
P values [2]	ns	< 0.01		ns	< 0.01	< 0.05	ns

[1] Mean ± SD.

[2] Probability that the observed difference between groups might occur by chance using Student's *t* test; ns indicates nonsignificance.

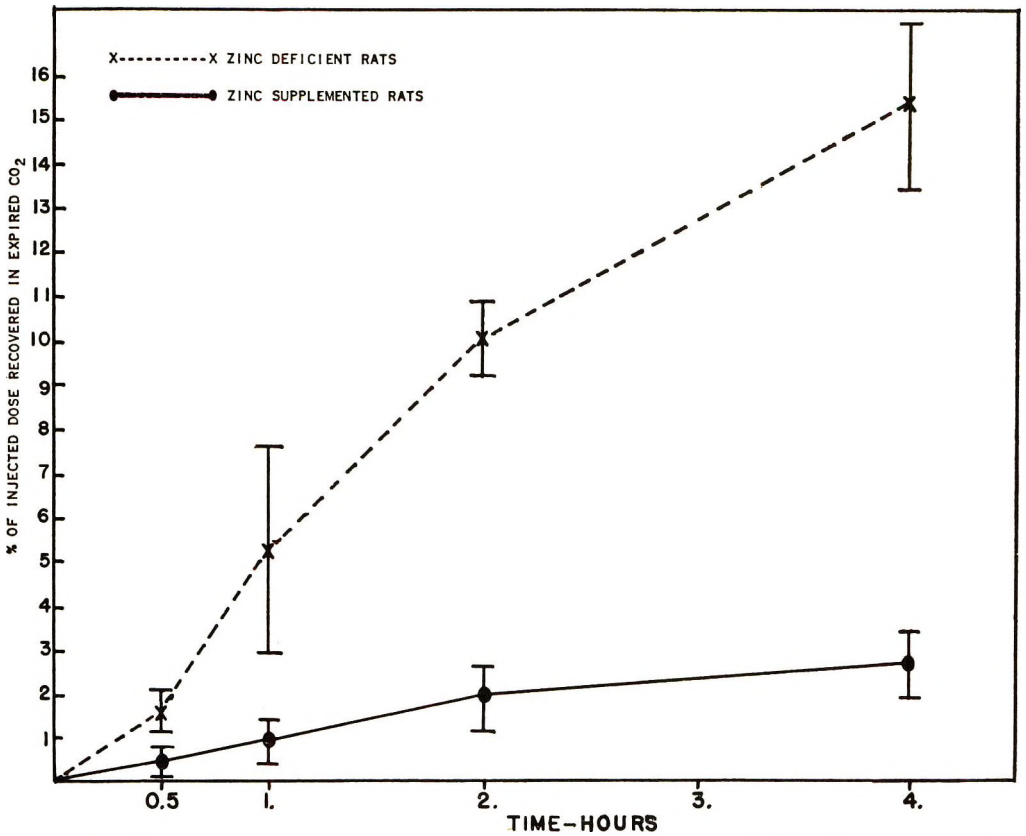

Fig. 1 Effect of zinc deficiency on oxidation of L-methionine methyl-^{14}C in rats after being fed experimental diets for 14 days. Each point is the average of five rats. The vertical lines are the standard deviations of the means.

in zinc-deficient rats was almost 10 times greater than in zinc-supplemented animals.

To determine whether the position of the label accounted for the differences, methionine labeled in the carboxyl group or in the second carbon was tested. The percentage of the methionine-1-^{14}C oxidized to CO_2 in 4 hours by zinc-deficient rats was higher than that by zinc-supplemented rats, but the difference was not statistically significant (table 3). Also, oxidation of methionine-2-^{14}C was not altered by zinc deficiency. These findings suggest that the methyl group is specifically involved during zinc deficiency.

The data in table 4 show that an intraperitoneal injection of nonradioactive DL-methionine (60 μmoles/100 g body weight) 10 minutes before L-methionine-methyl-^{14}C injection significantly increased the $^{14}CO_2$ formation in zinc-supplemented rats compared with those receiving no carrier dose. Only slight differences were found in the percentage oxidized in 3 hours between zinc-deficient rats with and without the injection of carrier dose. Therefore, the increased oxidation of L-methionine-methyl-^{14}C by zinc-deficient rats appeared to be less affected by pool size.

The effect of zinc repletion on the oxidative process of tracer methionine was determined in two separate trials (table 5). A single injection of 400 μg zinc did decrease $^{14}CO_2$ production, but the same dose given for 3 consecutive days was apparently needed to restore the oxidation of L-methionine-methyl-^{14}C to normal. Thus, the increased oxidation of tracer methionine by zinc-deficient rats was readily reversible. Furthermore, the fact that the mean body weight of the zinc-deficient rats remained unchanged after repletion indicated that oxidation of methionine was probably independent of animal size.

TABLE 3

Cumulative percentage of methionine-1-^{14}C and methionine-2-^{14}C converted to $^{14}CO_2$ by zinc-supplemented and zinc-deficient rats

Type of diet	Compound used	No. of rats	Days on exp. diet	Avg body wt	Hours			
					0.5	1	2	3	4
				g					
Zinc supplemented	Methionine-1-^{14}C	5	22	145 ± 27 [1]	0.76 ± 0.26	3.52 ± 0.75	8.93 ± 1.68	12.93 ± 1.89	15.93 ± 1.88
Zinc deficient	Methionine-1-^{14}C	5	22	84 ± 10	1.59 ± 1.13	6.52 ± 4.34	14.87 ± 8.50	20.82 ± 10.58	24.11 ± 11.73
Zinc supplemented	Methionine-2-^{14}C	4	16	110 ± 13	0.72 ± 0.51	2.72 ± 1.35	7.74 ± 2.76	10.98 ± 3.53	14.30 ± 4.74
Zinc deficient	Methionine-2-^{14}C	4	16	67 ± 4	1.12 ± 0.77	3.26 ± 1.80	7.20 ± 3.42	11.23 ± 4.77	14.36 ± 5.60

[1] Mean ± SD.

The effects of dietary zinc deprivation on the oxidation of ^{14}C-labeled acetate and other amino acids are summarized in table 6. More than 55% of the tracer dose of acetate was oxidized in 3 hours by zinc-supplemented and zinc-deficient rats. Similarly, zinc deficiency had little or no effect on glutamic acid oxidation. In contrast to the acetate and glutamic acid, significantly higher percentages of leucine-UL-^{14}C and leucine-1-^{14}C were converted to respiratory CO_2 in 2 and 6 hours, respectively, by zinc-deficient animals. These observations are in agreement with the results reported by other investigators (5). The data given in table 6 also show that zinc deficiency resulted in an increased oxidation of glycine-1-^{14}C, cystine-1-^{14}C and tryptophan-2-^{14}C.

To test whether the observed increase in the oxidation of L-methionine-methyl-^{14}C, tryptophan-2-^{14}C, glycine-1-^{14}C and cystine-1-^{14}C is mediated through a common mechanism stimulating the formate pathway, formaldehyde-^{14}C and formate-^{14}C were used and expired $^{14}CO_2$ determined. The results given in table 7 indicate that oxidation of formaldehyde was significantly increased by zinc deficiency. The primary difference between zinc-supplemented and zinc-deficient rats in the percentage of the dose oxidized in 3 hours occurred in the first half hour of $^{14}CO_2$ collection. Formate oxidation was unaffected by zinc deficiency.

DISCUSSION

Increased oxidation of UL-^{14}C-labeled leucine and lysine in zinc-deficient rats was first recognized by Theuer and Hoekstra (5). This metabolic defect was restored to normal by feeding a zinc-supplemented diet. Mills et al. (2), however, were unable to show any significant changes in $^{14}CO_2$ output when UL-^{14}C-labeled *Chlorella* protein was fed to zinc-deficient rats. Data presented here indicate that zinc-deficient animals converted methyl-labeled methionine to respiratory $^{14}CO_2$ at a rate 5 to 10 times faster than control rats. To a lesser extent, oxidation of leucine-1-^{14}C, glycine-1-^{14}C, cystine-1-^{14}C, tryptophan-2-^{14}C and formaldehyde-^{14}C were also significantly increased during zinc deficiency. The specific involvement of zinc with the methyl group of methionine is indicated by the findings

TABLE 4

Recovery of L-*methionine-methyl-*^{14}C *as* $^{14}CO_2$ *in rats fed zinc-supplemented and zinc-deficient diets*

Type of diet	No. of rats	DL-Methionine carrier, 60 μmole/100 g	Days on exp. diet	Avg body wt	Three-hour cumulative recovery	P values [1]
				g	*% injected dose*	
Zinc supplemented	6	—	22	121 ± 3 [2]	3.06 ± 0.39	< 0.01
Zinc deficient	7	—	22	58 ± 3	18.20 ± 0.45	
Zinc supplemented	5	+	14	93 ± 5	10.85 ± 0.68	< 0.01
Zinc deficient	5	+	14	57 ± 4	21.57 ± 4.31	

[1] Probability that the observed difference between groups might occur by chance using Student's *t* test.
[2] Mean ± SD.

TABLE 5

Recovery of L-*methionine-methyl-*^{14}C *as* $^{14}CO_2$ *by zinc-repleted rats*

Experiment no.	Type of diet	No. of rats	Final body wt, 14 days	Three-hour cumulative recovery	P values [1]
			g	*% injected dose*	
1	Zinc supplemented	3	109 ± 7 [2]	1.96 ± 0.12	< 0.05
	Zinc deficient and repleted [3]	3	60 ± 5	4.31 ± 1.13	
2	Zinc supplemented	5	97 ± 4	1.43 ± 0.48	< 0.01
	Zinc deficient	3	54 ± 2	10.13 ± 1.97	
	Zinc deficient and repleted [4]	5	66 ± 19	2.62 ± 1.68	< 0.01

[1] Probability that the observed difference between groups might occur by chance using Student's *t* test. In experiment 2, no statistical significance was found between zinc-supplemented and zinc-repleted groups.
[2] Mean ± SD.
[3] Zinc-deficient rats received a single intraperitoneal injection of 400 μg zinc as $ZnCl_2$ 1 hour before isotope injection.
[4] Zinc-deficient rats received intraperitoneal injections of 400 μg zinc as $ZnCl_2$ daily for 3 consecutive days.

TABLE 6

Recovery of ^{14}C*-labeled acetate and amino acids as* $^{14}CO_2$ *by zinc-supplemented and zinc-deficient rats*

Type of diet	No. of rats [1]	Substrates	Hours after injection	Cumulative recovery	P values [2]
				% injected dose	
Zinc supplemented	5	Acetate-2-^{14}C	3	56.1 ± 1.15 [3]	ns
Zinc deficient	4		3	54.2 ± 1.08	
Zinc supplemented	3	DL-Tryptophan-2-^{14}C	3	2.75 ± 0.12	< 0.01
Zinc deficient	3		3	6.68 ± 1.14	
Zinc supplemented	4	DL-Glutamic-1-^{14}C	3	59.8 ± 1.74	ns
Zinc deficient	4		3	56.3 ± 3.21	
Zinc supplemented	5	L-Leucine-(UL)-^{14}C	2	7.7 ± 0.12	< 0.01
Zinc deficient	5		2	15.1 ± 1.81	
Zinc supplemented	5	L-Leucine-1-^{14}C	6	18.1 ± 0.22	< 0.01
Zinc deficient	5		6	40.4 ± 1.36	
Zinc supplemented	4	Glycine-1-^{14}C	2	14.9 ± 2.51	< 0.05
Zinc deficient	4		2	24.9 ± 5.21	
Zinc supplemented	4	DL-Cystine-1-^{14}C	1	7.1 ± 0.51	< 0.01
Zinc deficient	4		1	14.8 ± 2.61	

[1] All rats on experimental diet for 14 days.
[2] Probability that the observed difference between groups might occur by chance using Student's *t* test; ns indicates nonsignificance.
[3] Mean ± SD.

TABLE 7

Cumulative percentage of formaldehyde-^{14}C and sodium formate-^{14}C converted to $^{14}CO_2$ by zinc-supplemented and zinc-deficient rats

Type of diet	Compound used	No. of rats	Days on exp. diet	Avg body wt	Hours			
					0.5	1	2	3
				g				
Zinc supplemented	Formaldehyde-^{14}C	5	14	104 ± 13.6 [1]	11.49 ± 1.51	26.21 ± 1.94	37.58 ± 1.80	42.18 ± 1.55
Zinc deficient		5	14	65 ± 4.6	17.90 ± 2.76	31.00 ± 1.36	40.96 ± 2.70	45.84 ± 2.98
P value [2]				< 0.01	< 0.01	< 0.01	< 0.01	< 0.05
Zinc supplemented	Formate-^{14}C	5	20	125 ± 6.3	14.40 ± 4.31	26.83 ± 4.71	36.93 ± 3.38	40.84 ± 3.10
Zinc deficient		5	20	72 ± 5.2	17.45 ± 3.38	29.42 ± 3.84	38.58 ± 3.01	43.16 ± 2.66
P value				< 0.01	ns	ns	ns	ns

[1] Mean ± SD.
[2] Probability that the observed difference between groups might occur by chance using Student's *t* test; ns indicates nonsignificance.

of normal oxidation of methionine-1-^{14}C and methionine-2-^{14}C in the zinc-deficient rats.

The question that remains to be answered is whether the increased $^{14}CO_2$ expiration after injection of L-methionine-methyl-^{14}C into zinc-deficient animals is actually a reflection of an increased rate of oxidation of this compound, or whether it is influenced by differences in intracellular amino acid pools. Data shown in table 4 indicate that zinc-supplemented rats receiving a carrier dose of methionine have an increased respiratory $^{14}CO_2$ output when compared with those without carrier. No such effect was noted in zinc-deficient animals. Thus, it appears that alterations in methionine pool size affects the oxidation of its methyl group more in zinc-supplemented rats than in zinc-deficient animals. Nevertheless, this finding does not rule out the possibility of a relationship between methyl group oxidation and changes in pool size.

The specific activity of pancreatic protein isolated from the trichloroacetic acid-soluble fraction was higher in zinc-deficient rats than in rats fed zinc, 0.5 and 2 hours after L-methionine-methyl-^{14}C injection. No differences were found in the specific activity of the liver, kidney or plasma. When carrier doses were used, however, the increased specific activity of pancreatic protein previously observed was abolished and the specific activities of the liver, kidney and plasma were significantly decreased.[6] These findings led us to believe that zinc is somewhat involved in protein metabolism.

Another possibility is a requirement of zinc in transmethylation. Any defect in the pathway from the methyl group to one or more of its acceptors could ultimately increase the oxidation of methyl groups to respiratory CO_2. The observations (table 7) that zinc-deficient rats had a greater rate of formaldehyde oxidation would suggest that increased oxidations of certain labeled amino acids during zinc deficiency are, in part, linked to the formate pathway. The conversion of formaldehyde to formic acid is catalyzed by the enzyme formaldehyde dehydrogenase which specifically requires

[6] J. M. Hsu, W. L. Anthony and P. J. Buchanan 1968 unpublished data.

reduced glutathione (GSH) as cofactor (6). Kuchinskas (7) has demonstrated that in vitro, addition of GSH to the rat liver enzyme preparation gave a pronounced stimulation in oxidation of the methyl group of L-methionine to CO_2. Thus, the increased liver GSH synthesis in zinc-deficient rats (8) might be partly responsible for the enhancement of oxidative breakdown of L-methionine-methyl-^{14}C. Knowledge concerning the effect of zinc deficiency on formaldehyde dehydrogenase activity is needed to test the validity of this interpretation.

On the other hand, the increased leucine oxidation by zinc deficiency still requires a different explanation since the known metabolic fate of this amino acid does not involve formaldehyde or formate. The nature of the relationship between zinc deficiency and amino acid oxidation thus remains to be elucidated. Additional studies in vitro will also be needed to determine whether amino acid oxidation occurs at an increased rate in all tissues of zinc-deficient rats, or is restricted to the pancreas, the zinc content of which is drastically decreased.

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Factors in Whole-egg Protein Influencing Dietary Induction of Increases in Enzyme and RNA Levels in Rat Pancreas [1]

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ABSTRACT Protein constituents of whole-egg powder responsible for the induction of increased amounts of chymotrypsinogen, trypsinogen, amylase lipase and RNA in the pancreas were studied in rats fed two meals per day and killed while in the post-absorptive state. Improvement of the protein quality of a 15% casein diet with addition of methionine or 15% more casein raised enzyme but not RNA levels in the pancreas. Addition of egg white trypsin inhibitor (EWTI) raised enzyme and RNA levels. Egg protein, heated to destroy EWTI, and an amino acid mixture simulating whole-egg protein were not equivalent to unheated egg protein. EWTI did not induce increases in pancreatic components when fed with an amino acid diet. In contrast to EWTI, soybean trypsin inhibitor (SBTI) fed with a casein diet depressed amylase in the pancreas although it elevated levels of chymotrypsinogen, trypsinogen and lipase. Chymotrypsinogen was increased significantly more by SBTI than by EWTI. The enzyme pattern produced in the pancreas of rats injected with pancreozymin three times daily for 1 week was similar to that produced by feeding EWTI for 1 week. Pancreozymin did not induce increases in pancreatic RNA.

Substitution of whole-egg protein for casein in a semipurified diet causes the pancreatic content of RNA, amylase, trypsinogen, chymotrypsinogen, and lipase to increase in rats. Adaptation to whole-egg protein begins almost immediately and is complete within 3 to 7 days (1).

The ability of whole-egg protein to induce changes in the composition of the exocrine pancreas may be related to the superior amino acid composition of this protein and to its content of egg white trypsin inhibitor (EWTI). Vandermeers et al. (2) showed that exportable enzymes are reduced in the pancreas when a poor quality protein such as gluten is fed. Deficiencies or excesses of an amino acid produce changes in pancreatic enzyme levels with some enzymes being affected more than others by a particular excess or deficiency (3). According to results obtained in previous studies, however, the pancreatic content of chymotrypsinogen and trypsinogen of rats fed casein and hydrolyzed casein diets is similar, but hydrolysis of egg diminishes its ability to induce increased chymotrypsinogen and trypsinogen (4). Therefore, one must conclude that the pancreatic response to whole-egg protein is in part a response to one or more individual egg proteins, whose biological action must be exerted in the gastrointestinal tract before the protein is completely hydrolyzed.

One egg protein which may exert an effect on the pancreas is egg white trypsin inhibitor (EWTI). EWTI has not been studied as extensively as soybean trypsin (SBTI). Various investigators (5–7) have attributed to SBTI or raw soybean meal, when ingested, the ability to 1) cause pancreatic hypertrophy and hyperemia, 2) stimulate an excessive release of pancreatic enzymes and 3) increase the conversion of methionine to cystine in the pancreas.

Three hypotheses were tested in the experiments described below: 1) that the effects of EWTI and SBTI on the pancreas are similar; 2) that the pancreatic response to whole-egg protein, as evidenced by an increase in pancreatic enzymes and RNA, is a response to the superior amino acid composition of this protein and to its content of EWTI; and 3) that the effect of the two inhibitors is mediated through pancreozymin.

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TABLE 1

Composition of basal diet

Ingredient	g/100 g diet
Cellulose	5
Cottonseed oil	5
Salt mix [1]	4
Vitamin mix in sucrose [2]	1
Vitamins in cottonseed oil [3]	0.058
Protein source	variable
Sucrose	70 or less depending on amount of protein in diet

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

[2] Water-soluble vitamins were supplied in the diet at the following levels: (milligrams per kilogram) thiamine, 20; riboflavin, 20; pyridoxine, 14; Ca pantothenate, 140; inositol, 700; niacin, 140; folic acid, 1.8; biotin, 0.7; vitamin B_{12}, 0.12; choline chloride, 1500; and ascorbic acid, 700.

[3] Vitamins in oil supplied the following per kilogram diet: (in milligrams) *dl*-α-tocopherol, 49; and menadione, 0.08; (in International Units) vitamin A palmitate, 16,000; and calciferol, 2000.

EXPERIMENTAL PROCEDURE

Male albino rats of the Charles River CD strain, weighing 150 to 175 g, were fed 6.5 g of the basal diet (table 1) containing 15% casein twice a day for 1 week to adjust them to a semipurified diet and to the interval method of feeding. The rats were segregated into groups of five and were fed the experimental diets described below for each investigation during week 2 of each study. The rats were fed at 8 AM and 5 PM except on the last day of the study, when they were fed at 8 AM and 8 PM. They were killed by chloroform inhalation 12 hours after the 8 PM feeding.

Experiment 1. To test whether the effects of feeding EWTI and SBTI on the pancreas were similar, groups of rats were fed one of the following diets during week 2 of the experiment: 15% casein, 15% casein + 1% EWTI,[2] and 15% casein + 0.87% SBTI.[3] The trypsin-inhibitor diets were formulated such that each diet inhibited identical amounts of trypsin as determined by the assay described below.

Experiment 2. To determine if a 15% casein diet could be "improved" so that in effect it more closely resembled an egg protein diet, each group was fed one of the following during week 2 of the experiment: 15% casein, 15% casein + 0.6% EWTI,[4] 15% casein + 0.2% *dl*-methionine, 15% casein + 0.2% *dl*-methionine + 0.6% EWTI, 30% casein + 0.6% EWTI, 30% casein and 18.6% hexane-extracted whole-egg powder. The 18.6% whole-egg powder diet provided the same amount of nitrogen as the 15% casein diet and contained about 0.9% EWTI according to the results of a subsequent analysis of egg powder from the same lot; hence, the amount of EWTI added to the casein diets was not equivalent to that in egg.

Experiment 3. To test whether the ability of egg protein to stimulate the pancreas was related to amino acid content or to the biological action of an intact protein contained in egg, rats were given the following experimental diets for 1 week: 18.6% unheated hexane-extracted whole-egg powder, 18.6% heated egg powder, 18.6% heated egg powder + 0.9% EWTI, 15% amino acids formulated to simulate whole-egg protein,[5] and 15% amino acids (egg pattern) + 0.9% EWTI. The amino acid and the egg powder diets were prepared to contain approximately the same amount of nitrogen. The denatured egg powder was prepared by boiling whole-egg powder in distilled water for 30 minutes. The solution was then freeze-dried.

Experiment 4. To determine if factors affecting the pancreas reside in the yolk, the white, or both, each group of rats was fed one of the following diets: 20.6% hexane-extracted whole-egg powder, 20.6% whole-egg powder + 1.4% EWTI,[6] 18.6% spray dried egg white solids, 22.6% hexane-extracted egg yolk or 22.6% hexane-extracted egg yolk + 1.2% EWTI. The diets without added inhibitor were formulated to contain 15% protein according to an analysis of each lot of protein. According to the assay outlined below for EWTI, the inhibitor content of the five diets, as analyzed, was as follows: whole-egg, 1.2%; whole-egg + EWTI, 2.6%; egg white, 2.8%; egg yolk, 0%; and egg yolk + EWTI, 1.2%.

Experiment 5. The hypothesis that the action of pancreozymin is similar to that of EWTI was tested. Groups of rats were fed 15% casein during week 2 of the ex-

[2] EWTI and SBTI were purchased from Sigma Chemical Company, St. Louis, Mo.

[3] See footnote 2.

[4] EWTI purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

[5] Orr, M. L., and B. K. Watt 1957 Amino Acid Content of Foods. Home Econ. Res. rep. no. 4, USDA.

[6] See footnote 4.

periment and, in addition, were given subcutaneous injections three times per day (8 AM, 12 PM and 4 PM) of one of the following substances: 0.9% NaCl, 2 Crick units of pancreozymin [7] or 4 Crick units of pancreozymin.

Preparation of pancreas samples. After removal, each pancreas was freed, as much as possible, from surrounding fat and connective tissue and then was homogenized in ice-cold 0.25 M sucrose solution for 2 minutes at 30,000 rpm in a homogenizer.[8] The final volume of the homogenate was 10 ml. Aliquots of the exact size needed for the determinations to be run were pipetted into test tubes and frozen for 2 to 6 weeks. Analysis of each specific pancreatic component was usually completed within 5 days. It had been previously determined that the outcome of the analyses to be run was not affected by the length of the freezing period.

Chemical analyses. For analysis of chymotrypsinogen, trypsinogen and amylase, frozen aliquots containing about 100 mg wet pancreas were thawed and made up to a final volume of 10 ml with 0.2 M Tris buffer (pH 8.1), containing 0.05 M $CaCl_2$. Fifteen milligrams of purified enterokinase were added to activate chymotrypsinogen and trypsinogen. The activation step was carried out at 4° for 18 to 24 hours. Particulate matter settled during this time period; enzyme assays were run using aliquots of the supernatant. Chymotryptic and tryptic activities were estimated using *N*-acetyltyrosine ethyl ester (ATEE) and *p*-toluene sulfonyl-L-arginine methyl ester (TAME), respectively. The two assays, as described in detail in a previous paper (8), are accomplished by adding 1 ml diluted, activated pancreatic homogenate to 2 ml buffer-substrate-pH indicator solution (0.02 M ATEE or TAME, 0.015 M Tris buffer (pH 8.1); 0.03 M $CaCl_2$; and 0.015% *m*-nitrophenol) and by measuring the rate at which hydrogen ion is released during hydrolysis of the esters at 395 mμ using a spectrophotometer.[9] Amylase was assayed by the starch-iodine method of Smith and Roe (9).

A modification of the method of Guth et al. (10) was used to assay for lipase. An aliquot of pancreatic homogenate was diluted with 0.9% NaCl so that a sample containing approximately 0.5 mg wet pancreas could be obtained. This sample was added to a substrate solution consisting of 0.08% emulsified corn oil, 0.75% sodium deoxycholate, 0.004% potassium oleate and 3% gelatin in 0.1 M Tris buffer (pH 8.1). Activity was determined by measuring the rate at which the turbidity of the solution disappeared at 30° using a spectrophotometer [10] set at 530 mμ. Values for lipase assayed on the Gilford (exps. 1, 4 and 5) tended to be lower than corresponding values obtained using the Spectronic 20.

Trypsin inhibitor activity was determined as follows: 10 μg trypsin, dissolved in a solution of 0.015 M Tris buffer (pH 8.1) and 0.03 M $CaCl_2$, were mixed by agitating for about 30 seconds with a solution containing zero to 10 μg of SBTI or EWTI dissolved in distilled water. Insoluble proteins or other substances were removed by centrifugation from inhibitor preparations made from egg protein or prepared diets. The mixture of trypsin and inhibitor was brought to a final volume of 1 ml, and the tryptic activity of the solution was determined by the method described above using 2 ml TAME assay solution. The tryptic activity of the mixture was plotted against micrograms of pure inhibitor, egg protein or diet added. The amount of pure inhibitor, egg protein or diet required to inhibit 10 μg of trypsin was determined by extrapolation. Thus, the inhibitor content of a protein or diet mixture could be determined by comparing the amount of these required to inhibit 10 μg of trypsin with the amount of pure inhibitor required.

The RNA content of a 200-mg sample of wet pancreas was determined by a magnification of the Schneider (11) technique.

RESULTS

Comparison of EWTI and SBTI diets. Data shown in tables 2 and 3 demonstrate that the effects of SBTI and EWTI on pancreatic enzymes of rats in the postabsorptive state are not identical. For example, chymotrypsinogen, the activity of which is expressed for comparison in four ways in

[7] Sigma Chemical Company, St. Louis.
[8] Virtis Model 45, Virtis Company, Gardiner, N. Y.
[9] Beckman B, Beckman Instruments, Inc., Fullerton, Calif.
[10] Bausch and Lamb Spectronic 20, Bausch and Lomb, Rochester, N. Y.; or Gilford model 240, with automatic cuvette changer, Gilford Instrument Laboratories Inc., Oberlin, Ohio.

TABLE 2

Effect of EWTI and SBTI diets on chymotrypsinogen content (expressed in four manners) of pancreas of rats in the postabsorptive state (exp. 1)

Diet	mmoles ATEE hydrolyzed per minute			
	per pancreas	*per g pancreas*	*per mg pancreatic DNA*	*per pancreas per 100 g body wt*
Casein	1.37 ± 0.23 [a,1]	1.36 ± 0.14 [a]	0.48 ± 0.06 [a]	0.59 ± 0.10 [a]
Casein + 1% EWTI	2.17 ± 0.18 [b]	2.11 ± 0.18 [b]	0.66 ± 0.07 [a]	0.90 ± 0.07 [b]
Casein + 0.87% SBTI	4.42 ± 0.29 [c]	3.51 ± 0.14 [c]	1.10 ± 0.07 [b]	1.95 ± 0.15 [c]

[1] Mean ± SE. Means without a common letter in their superscript are significantly different at $P < 0.05$.

TABLE 3

Effect of EWTI and SBTI diets on weight and enzyme content of pancreas of rats in the postabsorptive state (exp. 1)

Diet	Pancreas wt	Trypsinogen	Amylase	Lipase
	g	*mmoles TAME/ min per pancreas*	*g starch/ min per pancreas*	*g corn oil/ min per pancreas*
Casein	0.98 ± 0.07 [a,1]	0.39 ± 0.05 [a]	4.70 ± 0.57 [a]	0.30 ± 0.04 [a]
Casein + 1% EWTI	1.03 ± 0.04 [a]	0.72 ± 0.10 [b]	7.40 ± 1.02 [b]	0.38 ± 0.02 [a]
Casein + 0.87% SBTI	1.26 ± 0.08 [b]	0.89 ± 0.11 [b]	3.04 ± 0.64 [a]	0.36 ± 0.03 [a]

[1] Mean ± SE. Means without a common letter in their superscript are significantly different at $P < 0.05$.

table 2, increased in the pancreas when either SBTI or EWTI was added to the 15% casein diet. Significantly more chymotrypsinogen, however, was found in the pancreas when SBTI was fed compared with EWTI. The values tabulated in table 2 also demonstrate that in studies of the type reported here, interpretation of pancreatic data is not dependent on the manner in which the data are expressed. Hence, enzyme data will be reported below as total activity per pancreas. As mentioned in a previous paper (1), this method of expressing data is not satisfactory when rats of different age or size are compared.

Addition of EWTI and SBTI to the 15% casein diet also acted to increase levels of trypsinogen in the pancreas (table 3). However, whereas amylolytic content was enhanced by addition of EWTI to the diet, SBTI added to the diet depressed amylolytic content. Lipase was not significantly affected by either treatment. Pancreas weight increased significantly when SBTI, but not EWTI was fed.

Comparison of casein and whole-egg protein diets. Whether a 15% casein diet could be "improved" so that its effect on the pancreas mimicked that of whole-egg protein was tested in experiment 2. The effect of adding methionine, of doubling the casein content of the diet or of feeding EWTI with casein is compared in table 4 with the effect of feeding whole-egg protein. Rats receiving the 15% casein control diet, had the lowest levels of pancreatic enzymes. The substitution of the 30% casein diet for the 15% casein diet significantly raised levels of all four enzymes. The addition of methionine caused chymotrypsinogen and amylase to increase significantly. Although trypsinogen and lipase did not increase significantly when methionine was added to the casein diet, levels of these enzymes were elevated such that they did not differ significantly from levels measured in rats fed whole-egg protein. Addition of EWTI to the diets containing 15% casein and 30% casein tended to induce increases in the enzyme content of the pancreas. With the exception of lipase, corresponding increases were not induced by the addition of EWTI to the diet containing 15% casein and free methionine. Specific treatments did not affect all enzymes equally. For example, chymotrypsinogen increased dramatically in rats fed 30% casein + EWTI, whereas amylase was most affected by the addition of methionine to the casein diet.

TABLE 4

Comparison of response of pancreatic enzymes and RNA to whole-egg protein diet or casein diets altered to increase protein quality, quantity or trypsin inhibitor content (exp. 2)

Dietary treatment	Chymotrypsinogen	Trypsinogen	Amylase	Lipase	RNA
	mmoles ATEE/min per pancreas	*mmoles TAME/min per pancreas*	*g starch/min per pancreas*	*g corn oil/min per pancreas*	*mg/pancreas*
15% casein	1.23 ± 0.19 [a,1]	0.53 ± 0.07 [a]	3.35 ± 0.56 [a]	0.28 ± 0.03 [a]	16.3 ± 1.0 [a]
15% casein + 0.6% EWTI	2.29 ± 0.51 [abc]	0.73 ± 0.08 [ab]	5.17 ± 1.07 [ab]	0.53 ± 0.13 [a]	20.2 ± 0.8 [bc]
15% casein + 0.2% methionine	2.56 ± 0.14 [bc]	0.68 ± 0.08 [ab]	8.79 ± 1.13 [c]	0.60 ± 0.17 [ab]	17.0 ± 1.5 [ab]
15% casein + 0.2% methionine + 0.6% EWTI	2.28 ± 0.12 [b]	0.62 ± 0.04 [a]	7.24 ± 0.66 [bc]	0.96 ± 0.11 [bc]	21.2 ± 1.2 [bcd]
30% casein	3.05 ± 0.30 [c]	0.86 ± 0.06 [bc]	7.17 ± 1.23 [bc]	1.04 ± 0.11 [bc]	18.0 ± 1.2 [abc]
30% casein + 0.6% EWTI	4.43 ± 0.32 [d]	1.05 ± 0.09 [c]	7.66 ± 1.44 [bc]	1.00 ± 0.07 [c]	22.8 ± 1.7 [cd]
18.6% whole-egg powder [2]	2.61 ± 0.23 [bc]	0.97 ± 0.22 [bc]	7.74 ± 1.69 [bc]	0.86 ± 0.04 [bc]	23.6 ± 0.7 [d]

[1] Mean and SE. Means without a common letter in their superscripts are significantly different at $P < 0.05$.
[2] Whole-egg powder diet was formulated to contain the same amount of protein as the casein diet.

An interesting observation made in experiment 2 was that the presence of EWTI in any of the diets fed caused RNA to increase in the pancreas. Pancreatic RNA was more responsive to additions of small amounts of EWTI than to any other change effected in the protein component of the diet.

Comparison of effects of components in egg protein. An attempt was made in experiment 3 to isolate more specifically factors in egg responsible for pancreatic stimulation. This was done by feeding heated egg powder, in which the activity of EWTI was destroyed by denaturation, and by feeding amino acid diets formulated to simulate whole-egg protein. Data shown in table 5 demonstrate that pancreatic levels of all four enzymes and of RNA were reduced at least 17% in rats fed denatured, as opposed to unheated, whole-egg protein. Addition of EWTI to the heated egg diet raised levels of all pancreatic components of interest, except amylase, from 7 to 55%. The diet containing heated egg + EWTI, however, tended to enhance pancreatic enzyme content to a lesser extent than did the unheated egg powder diet. Hence, the possibility cannot be excluded that one or more proteins in egg, in addition to EWTI, might have some special effect on the pancreas; also, the protein quality of the egg powder diet might have been reduced by heating.

Similar amounts of chymotrypsinogen, trypsinogen and lipase, but not amylase, were found in the pancreas of rats fed the heated egg and the amino acid diets. Levels of all enzymes except trypsinogen were lower when rats were fed the amino acid diet with EWTI compared with the amino acid diet without inhibitor.

Experiment 4 was performed to test if the factors responsible for inducing increases in enzyme and RNA content of the pancreas reside in the white, the yolk or in both fractions. The results shown in table 6 demonstrate again that EWTI is an important factor. Rats fed the inhibitor-free egg yolk diet had the lowest pancreatic levels of enzymes and RNA. Addition of 1.2% EWTI to the egg yolk or 1.4% EWTI to the whole-egg protein diet tended to raise levels of all pancreatic constituents measured. The diets that produced the

TABLE 5

Effect of feeding diets with whole-egg protein, denatured whole-egg protein or amino acids in egg pattern on pancreatic enzymes and RNA (exp. 3)

Dietary treatment	Chymotrypsinogen	Trypsinogen	Amylase	Lipase	RNA
	mmoles ATEE/ min per pancreas	*mmoles TAME/ min per pancreas*	*g starch/ min per pancreas*	*g corn oil/ min per pancreas*	*mg/pancreas*
Egg protein	$3.77 \pm 0.20^{a,1}$	1.10 ± 0.08^{a}	9.36 ± 1.22^{a}	0.99 ± 0.11^{a}	25.1 ± 1.43^{a}
Egg protein (heated)	2.67 ± 0.20^{b}	0.87 ± 0.07^{ab}	7.72 ± 0.26^{a}	0.52 ± 0.06^{b}	20.9 ± 0.48^{b}
Egg protein (heated) + 0.9% EWTI	3.94 ± 0.36^{a}	0.99 ± 0.08^{ab}	7.53 ± 1.01^{a}	0.81 ± 0.18^{ab}	22.4 ± 0.72^{ab}
Amino acids (egg pattern)	2.59 ± 0.24^{b}	0.76 ± 0.08^{b}	4.61 ± 0.62^{b}	0.65 ± 0.07^{b}	19.7 ± 0.18^{c}
Amino acids (egg pattern) + 0.9% EWTI	2.27 ± 0.17^{b}	0.88 ± 0.10^{ab}	3.51 ± 0.53^{b}	0.61 ± 0.02^{b}	20.8 ± 0.82^{bc}

[1] Mean ± SE. Means without a common letter in their superscripts are significantly different at $P < 0.05$.

TABLE 6

Effect of feeding various proteins in egg on pancreatic enzymes and RNA (exp. 4)

Dietary protein	Chymotrypsinogen	Trypsinogen	Amylase	Lipase	RNA
	mmoles ATEE/ min per pancreas	*mmoles TAME/ min per pancreas*	*g starch/ min per pancreas*	*g corn oil/ min per pancreas*	*mg/pancreas*
Whole-egg	$3.74 \pm 0.31^{a,1}$	0.96 ± 0.07^{a}	7.79 ± 1.39^{a}	0.41 ± 0.07^{a}	23.4 ± 2.3^{ab}
Whole-egg + 1.4% EWTI	4.10 ± 0.53^{ab}	1.21 ± 0.13^{ab}	8.46 ± 1.78^{a}	0.44 ± 0.07^{a}	26.5 ± 0.8^{a}
Egg white	5.25 ± 0.51^{b}	1.43 ± 0.06^{b}	8.08 ± 1.69^{a}	0.47 ± 0.05^{a}	25.9 ± 1.5^{ab}
Egg yolk	2.66 ± 0.19^{c}	0.90 ± 0.08^{a}	4.64 ± 1.55^{a}	0.32 ± 0.06^{a}	20.5 ± 2.0^{b}
Egg yolk + 1.2% EWTI	2.79 ± 0.26^{c}	0.99 ± 0.09^{a}	8.67 ± 1.60^{a}	0.40 ± 0.08^{a}	24.2 ± 1.4^{ab}

[1] Mean ± SE. Means without a common letter in their superscripts are significantly different at $P < 0.05$.

TABLE 7

Effect of pancreozymin on enzyme and RNA content of pancreas of rats in the postabsorptive state (exp. 5)

Treatment	Chymotrypsinogen	Trypsinogen	Amylase	Lipase	RNA
	mmoles ATEE/min per pancreas	*mmoles TAME/min per pancreas*	*g starch/min per pancreas*	*g corn oil/min per pancreas*	*mg/pancreas*
Saline injections	1.56 ± 0.13 [a,1]	0.64 ± 0.07 [a]	4.33 ± 0.93 [a]	0.29 ± 0.02 [a]	15.1 ± 0.8 [a]
Pancreozymin (2 units), 3 times/day	1.96 ± 0.21 [a]	0.61 ± 0.09 [a]	6.02 ± 1.55 [a]	0.35 ± 0.03 [ab]	14.3 ± 1.0 [a]
Pancreozymin (4 units), 3 times/day	2.78 ± 0.15 [b]	0.98 ± 0.09 [b]	8.27 ± 1.65 [a]	0.45 ± 0.05 [b]	13.0 ± 1.1 [a]

[1] Mean ± SE. Means without a common letter in their superscript are significantly different at $P < 0.05$.

most RNA were those containing the most EWTI.

Effect of pancreozymin on pancreas composition. Since presumably the site of action of EWTI and SBTI is the intestine, there is a possibility that the effect of these inhibitors on the pancreas is mediated in part through a hormone. This hormone might be pancreozymin. The effect of pancreozymin administration on pancreas composition was studied in experiment 5. Results, shown in table 7, demonstrate that pancreozymin, injected three times daily, tended to increase pancreatic enzyme levels and to lower levels of RNA. Concomitant with the administration of a larger dose of pancreozymin (12 rather than 6 units/day), was an increase in pancreatic enzyme levels. The effects of pancreozymin (exp. 5) and EWTI (exp. 1) were studied simultaneously although results of these experiments are reported separately in this paper. Comparison of data presented in tables 2, 3 and 7 demonstrates that the effects of pancreozymin and EWTI on the enzyme content of the pancreas were not dissimilar, although these substances apparently had opposite effects on pancreatic RNA.

DISCUSSION

Evidence collected in the experiments described here support the hypothesis that the pancreatic response to the dietary substitution of whole-egg protein for casein is essentially a response to the superior amino acid composition of egg and to its content of EWTI. Improvement of the protein quality of, or addition of EWTI to, a casein diet elevated enzyme levels in the pancreas of rats in the postabsorptive state. The principal factor in egg influencing pancreatic RNA, however, appears to be EWTI. To induce increases in pancreatic enzymes, EWTI apparently must interact with intact dietary protein, presumably in the small intestine, because this inhibitor does not enhance enzyme content when fed with an amino acid diet. The possibility cannot be excluded that there are other biologically active proteins in egg powder which might affect the pancreas.

The four enzymes of interest in these experiments (chymotrypsinogen, trypsinogen, amylase and lipase) usually were all

elevated or all depressed by some of the dietary manipulations performed, that is, improvement of the protein quality of a casein diet or feeding amino acids simulating egg protein rather than egg protein itself. Dietary-induced changes in pancreatic enzyme content, however, when parallel in direction were not always parallel in magnitude. For example, levels of chymotrypsinogen tended to be highest in rats fed 30% casein + EWTI and in those fed egg white protein. Amylase was increased more by the addition of methionine to the 15% casein diet than any other enzyme; conversely, amylase induction was depressed more than that of the other enzymes when amino acids, instead of intact egg, were fed. Trypsinogen and lipase often were not as responsive as chymotrypsinogen and amylase to dietary change.

Data obtained in these experiments are not entirely consistent with the hypothesis that the actions of EWTI and SBTI are similar. The most striking example of this was the observation that addition of EWTI to a 15% casein diet elevated, whereas addition of SBTI tended to depress, pancreatic amylase. This observation has been repeated in subsequent experiments.[11] The only instance in these experiments when a depression of amylase was accompanied by an increase in protease, particularly chymotrypsinogen, was when SBTI was fed with casein. It is interesting to note that there are only two other instances when this phenomenon has been shown to occur: 1) in rats made diabetic with alloxan (12), and 2) in rats switched from a high carbohydrate to a high protein diet (13). These observations, however, cannot be interpreted at this time as evidence that there is a relationship between the pancreatic response to SBTI and to a deficiency of insulin or glucose in the pancreas.

Several pieces of experimental evidence were considered prior to proposing that the action of trypsin inhibitors is mediated partially through pancreozymin. The first is that SBTI and EWTI apparently act as secretagogues, although Lepkovsky et al. (5) question some of the indirect evidence for this. The site of action of the inhibitors must be the intestine since their biological activity would be destroyed by digestion. Hence, one might assume that the effect of the inhibitors is mediated via a gastrointestinal hormone such as pancreozymin. Wang and Grossman (14) established that the release of pancreozymin is stimulated by the presence of protein and its digestion products in the small intestine. Third, according to Rothman and Wells (15), pancreozymin appears to enhance the synthesis, in a nonparallel fashion, of at least three exportable pancreatic enzymes.

The pancreozymin preparation given in experiment 5 may have contained up to 10% secretin. Rothman and Wells (15), however, observed that secretin administration to rats (20 U/kg body weight) produces only slight increases in pancreatic enzyme content; they attributed this effect to pancreozymin contamination. The only evidence obtained in these studies to support the hypothesis that the effect of EWTI on the pancreas is mediated through pancreozymin is indirect. The effects of both these substances on the pancreatic enzyme content of rats in the postabsorptive state are similar but not identical. Evidence unfavorable to this hypothesis includes the observation that EWTI, but not pancreozymin, causes an increase in pancreatic RNA However, the increase in pancreatic RNA but not in chymotrypsinogen, trypsinogen, amylase and lipase, can be prevented by injecting actinomycin D during the period of adaptation to a whole-egg protein diet (12). Therefore, the enzymic response to EWTI is apparently not dependent on an increase in total pancreatic RNA.

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Monodehydroascorbic Acid-Transhydrogenase Activity and Coenzyme Concentrations in Tissues of Ascorbic Acid-deficient and Control Guinea Pigs [1]

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ABSTRACT Adrenal gland and maternal placental tissue were assayed for NADH-monodehydroascorbic acid-transhydrogenase activity in a basic system containing ascorbic acid, NADH, KCN and microsomal protein in phosphate buffer, pH 7.4. Enzyme activity was not detectable when reduced glutathione and NADPH were substituted for ascorbic acid and NADH, respectively. The reaction was inhibited by *p*-chloromercuribenzoate. The average rate of NADH oxidation was higher in the adrenal gland than in the maternal placenta. Enzyme activity decreased as ascorbic acid concentration was decreased. NAD^+ and NADH concentrations were determined using an enzymatic method in adrenal glands of young male animals on ascorbic acid-deficient diets for 20 days, and in adrenal gland and maternal placenta of females on ascorbic acid-deficient diets for 15 days. Total NAD^+ and NADH concentrations were similar in ascorbic acid-deficient and control tissues whereas the average ratio of NAD^+/NADH was significantly depressed in the ascorbic acid-deficient tissues. The depressed ratio was attributed to a higher NADH concentration and generally lower NAD^+ concentration than was found in control tissues. Possible relationships of depressed NAD^+/NADH ratio and NADH-monodehydroascorbic acid-transhydrogenase activity are discussed in relation to known abnormalities of scurvy.

An ascorbic acid-dependent oxidation of NADH (reduced nicotinamide adenine dinucleotide) has been described in the microsomes of porcine adrenal gland (1), mouse liver (2) and bovine ocular tissue (3). The enzyme, NADH-monodehydroascorbic acid-transhydrogenase, was postulated to mediate the transfer of hydrogen from NADH to oxygen via the free radical intermediate, monodehydroascorbic acid, thus producing NAD^+ and regenerating ascorbic acid (1). Evidence for the participation of a free radical in ascorbic acid reactions has been obtained by Yamazaki et al. (4) using electron paramagnetic resonance spectroscopy.

The biological significance of this enzyme in microsomes is not clear. Ascorbic acid deficiency does lead to pronounced changes in collagen and mucopolysaccharide fractions of connective tissue (5) and in hormonal production (6). Dietary deficiency of ascorbic acid in the guinea pig causes a progressive decline in tissue concentrations of ascorbic acid. Conceivably, as the concentration of ascorbic acid declines below a critical level, the rate of NADH-monodehydroascorbic acid-transhydrogenase activity would also decline. The ensuing biochemical defect would be an accumulation of NADH and a change in the NAD^+/NADH ratio in the tissue. Conversion of pregnenolone to progesterone and corticosteroids has been shown to be inhibited by NADH (7). This inhibition was reversed by ascorbic acid. Several epimerase reactions involved in the formation of precursors for the synthesis of chondroitin sulfates require NAD^+ as a cofactor and inhibition of these reactions by NADH has been demonstrated in vitro (8–10). The biological significance of the ascorbic acid-dependent NADH oxidation may be to maintain the coenzyme in its oxidized form for participation in these reactions.

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In the present study the occurrence and characteristics of NADH-monodehydroascorbic acid-transhydrogenase were investigated in the adrenal gland and maternal placenta of the guinea pig. NAD^+ and NADH concentrations were determined in the same tissues obtained from ascorbic acid-deficient and control animals.

MATERIALS AND METHODS

Guinea pigs, from a colony derived from the Rockland Farms strain cross-bred with strain 13 from the National Institutes of Health, were used in all experiments. Animals were fed guinea pig ration [3] supplemented with fresh cabbage prior to the experimental periods. The experimental diet was an ascorbic acid-deficient diet prepared according to Krehl.[4] The diet was fed in pelleted form; water was given ad libitum. During the experimental period animals were weighed three times a week.

Male animals. Ten young male guinea pigs of the same age were paired according to weight and divided into two groups. Group 1 was fed the ascorbic acid-deficient diet for 20 days. Group 2 was pair-fed with the same diet to group 1 and given a daily oral supplement of 20 mg ascorbic acid. Animals were killed on day 20 of the experimental period.

Pregnant animals. Virgin female guinea pigs approximately 3 months of age were bred to male animals from the same colony. Copulation date was ascertained by making a vaginal smear on each animal with an open vagina. During the first 20 days of the 68-day gestation period, the guinea pigs were fed the stock diet. A supplement of fresh cabbage was given daily. After 20 days of gestation, the guinea pigs were paired according to weight, divided into two groups and treated as follows: group 1 was fed the ascorbic acid-deficient diet. Group 2 was pair-fed to group 1 using the same ascorbic acid-deficient diet and each animal was given a daily intraperitoneal injection of 10 mg of the sodium salt of ascorbic acid. All pregnant animals were killed on the morning of day 36 of gestation.

NADH-monodehydroascorbic acid-transhydrogenase activity. After decapitation, adrenal and maternal placental tissues were quickly removed and homogenized in cold 0.25 M sucrose. All procedures were carried out in an ice bath. Tissue homogenates were centrifuged in a preparative ultracentrifuge [5] at 4°. The supernatant fraction, obtained after centrifugation at 20,000 × *g* for 20 minutes, was centrifuged at 110,000 × *g* for 45 minutes to obtain a microsomal pellet.

All assays were done on fresh tissue immediately following killing. Enzyme activity was assayed by following NADH oxidation at 340 mμ on a spectrophotometer [6] using standard micro-silica cells, 1 cm light path. The basic system contained microsomal protein, 2.3×10^{-3} M ascorbic acid, 1.0 mM KCN and 5.0×10^{-5} M NADH in 0.06 M phosphate buffer, pH 7.4. The following variations in the basic system were tested: 1) NADPH was substituted for NADH at the same concentration; 2) reduced glutathione was substituted for ascorbic acid at the same concentration; 3) concentrations of ascorbic acid were varied from 2.3×10^{-2} M to 2.3×10^{-4} M; 4) concentrations of microsomal protein were varied; 5) iodoacetate, 10^{-3} M, was added to the basic system; 6) the sodium salt of *p*-chloromercuribenzoic acid, 10^{-4} M, was added to the basic system; and 7) ascorbic acid was added to the microsomal suspension in the absence of NADH.

Nicotinamide adenine dinucleotide determinations. Adrenal glands and maternal placental tissues were analyzed for NAD^+ and NADH by the fluorometric enzymatic cycling method of Lowry et al. (11). Tissues were removed within 3 minutes after decapitation, quickly rinsed, blotted and placed in cold homogenizing solution. Tissue preparation and homogenization procedures were carried out in an ice bath. Recovery of the coenzyme was studied by adding NAD^+ and NADH to tissue homogenates prior to incubation.

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[3] Rockland Guinea Pig Diet, purchased from R. and E. Feed Company, Troy, N. Y.

[4] Vitamin C-deficient guinea pig test diet prepared according to W. A. Krehl, Laboratory Manual at Yale Nutritional Laboratory, New Haven, Conn. Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. Composition of the diet as follows: (in percent) ground rolled oats, 40.0; wheat bran, 15.0; alfalfa leaf meal, 8.0; whole milk powder, 20.0; casein, 10.0; cottonseed oil, 5.0; sodium chloride, 0.5; calcium carbonate, 1.0; and magnesium sulfate, 0.5.

[5] Spinco, Model L, Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

[6] Beckman DU, Beckman Instruments, Inc., Fullerton, Calif.

Following the procedures suggested by Lowry et al. (11), tissues for NAD^+ analysis were homogenized in 0.1 mM KCN–0.01 M H_2SO_4–0.1 M Na_2SO_4; tissues for NADH analysis were homogenized in 0.1 mM KCN–0.02 N NaOH containing 0.5 mmole cysteine. Adrenal gland tissue was homogenized in a glass Potter-Elvehjem homogenizer; maternal placental tissues were minced and first homogenized in a mixer [7] and then with the glass Potter-Elvehjem homogenizer. As suggested by Lowry et al. (11) the samples for NADH determination were incubated at 60° for 10 minutes to destroy the oxidized form of the coenzyme and to retard oxidation of NADH by blood. The samples for NAD^+ determination were incubated for 45 minutes at 60° to retard NADase activity and to destroy the reduced form of the coenzyme.

Fluorescence was measured on a fluorometer,[8] with a primary filter (Corning no. 7–37) [9] and three secondary filters (Corning nos. 3–37, 5–61 and 4–70).[10] The fluorometer was adjusted before each assay with a quinine sulfate standard.

Mean differences between total coenzyme concentrations and NAD^+/NADH ratios in the ascorbic acid-deficient and control tissues were tested for significance by Student's *t* test (12).

Protein and ascorbic acid determinations. Protein was determined by the method of Lowry et al. (13). Total ascorbic acid was determined by the micromethod of Bessey et al. (14).

RESULTS

NADH-monodehydroascorbic acid-transhydrogenase. Enzyme activity was found in the adrenal tissue from young male animals, and in adrenal and maternal placental tissue from pregnant guinea pigs at day 35 of gestation. Results are presented for tissues obtained from pregnant animals (fig. 1). The enzyme activity was localized in the microsomal fraction. The initial velocity of the enzyme reaction was proportional to protein concentration within a range of 0.10 to 0.60 mg protein/400 µl. NADH was not oxidized when glutathione was substituted for ascorbic acid. NADPH, when substituted for NADH, was not oxidized by the enzyme. *p*-Chloromercuribenzoate completely inhibited the reaction, but sodium acetate caused only slight inhibition.

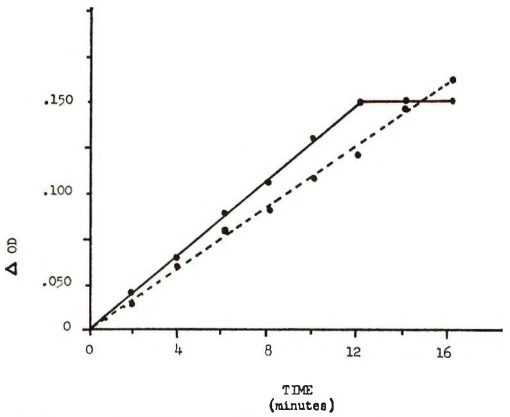

Fig. 1 Oxidation of NADH by adrenal gland (– – – –) and maternal placenta (——) microsomal preparations: 0.06 M phosphate buffer, pH 7.4; 37°; 1 mM KCN; 5×10^{-5} M NADH; 2.3×10^{-3} M ascorbic acid; microsomal protein: adrenal gland, 0.11 mg/400 µl and maternal placenta, 0.19 mg/400 µl.

Aghajanian (15) found that the addition of ascorbic acid to rat brain microsomes caused a decrease in optical density (OD) in the absence of NADH. This decrease was attributed to structural alterations in the microsomes. In the present experiments, no decrease in optical density was observed in either tissue when ascorbic acid was added to the microsomal suspension in the absence of NADH. The decrease in optical density observed in these experiments was due, therefore, to the oxidation of NADH and not to structural alterations of the microsomes.

The activity of the enzyme, expressed as change in OD per minute per milligram of protein, was higher in the adrenal gland than in the maternal placenta at all three ascorbic acid concentrations (table 1). The adrenal gland preparation in this study was about six times more active than a similar preparation from porcine microsomes reported by Staudinger et al. (1).

A point of inhibition was characteristic of all assays with maternal placental tissue (fig. 1). Ascorbic acid at a concentration of 2.3×10^{-2} M did not alleviate the

[7] Lourdes Model MM-1, Lourdes, Old Bethpage, N. Y.
[8] Farrand Model A, Farrand Optical Company, Inc., Mount Vernon, N. Y.
[9] Corning Glass Works, Medfield, Mass.
[10] See footnote 9.

TABLE 1

Activity of NADH-monodehydroascorbic acid-transhydrogenase in adrenal gland and maternal placenta of guinea pigs

Microsomes	Ascorbic acid concentrations		
	2.3×10^{-2} M	2.3×10^{-3} M	2.3×10^{-4} M
Adrenal gland	0.129 [1]	0.076	0.055
Maternal placenta	0.067	0.053	0.031

[1] Values are expressed as change in optical density per minute per milligram of protein after 12 minutes.

inhibition but at a lower concentration (2.3×10^{-4} M) inhibition occurred sooner. The inhibition is not due to a NADPH-transhydrogenase since no activity was observed when equal concentrations of NADPH and NAD^+ (total concentration 5.0×10^{-5} M) were substituted for NADH. The nature of this inhibition and its significance in the metabolism of the maternal placenta is being investigated.

The activity of the enzyme decreased as ascorbic acid concentrations were decreased (table 1). Using 2.3×10^{-3} M ascorbic acid as a reference point, adrenal gland activity was stimulated more with a 10-fold increase in concentration and depressed less with a 10-fold decrease in concentration than was the maternal placenta. When the ascorbic acid concentration was reduced to 2.3×10^{-5} M, conclusive evidence for enzyme activity could not be demonstrated in either tissue.

Tissues from ascorbic acid-deficient animals were also assayed. Using an ascorbic acid concentration of 2.3×10^{-3} M, no difference in enzyme activity could be demonstrated between ascorbic acid-deficient and control tissues. This finding shows that enzyme protein is not impaired in ascorbic acid deficiency. Enzyme activity without added ascorbic acid in vitro would not be expected since the conditions during isolation of the microsomes would favor the conversion of native ascorbic acid to dehydroascorbic acid. Staudinger et al. (1) have reported that dehydroascorbic acid cannot be substituted for ascorbic acid in the transhydrogenase reaction.

NAD^+ and NADH concentrations in adrenal gland. Male animals were killed after 20 days on the scorbutigenic diet to circumvent the effects of severe inanition. Ascorbic acid-deficient animals (group 1) gained weight at a rate equal to pair-fed controls up to day 13 and showed a slight drop in weight 2 days before killing. Animals in group 1 had hemorrhages in the knee joints at the time of killing with varying degrees of hemorrhages in the adrenal glands. Adrenal ascorbic acid concentration in ascorbic acid-deficient animals averaged 6.0 mg/100 g tissue as compared with 94.9 mg for pair-fed controls supplemented with ascorbic acid (table 2).

NAD^+ and NADH concentrations reported here are in the same range but higher than levels reported by other investigators in rat adrenal (16, 17). This difference probably reflects differences in species as well as differences in methods used for determination of NAD^+ and NADH.

Recovery studies were carried out on tissues from 5 of the 10 animals by adding NAD^+ and NADH to aliquots of the tissue homogenates prior to incubation. Recoveries ranged from 99 to 107% for NAD^+ and 99 to 106% for NADH.

Ascorbic acid-deficient animals in group 1 had an average NAD^+ plus NADH concentration of 8.01×10^{-13} moles/μg of fresh tissue with 3.86×10^{-13} moles/μg present in the oxidized form and 4.15×10^{-13} moles/μg present in the reduced form (table 2). The total NAD^+ plus NADH concentration of adrenal tissue of the control animals was 7.80×10^{-13} moles/μg of fresh tissue, with 4.54×10^{-13} moles/μg in the oxidized form and 3.26×10^{-13} moles/μg in the reduced form. The difference in total coenzyme concentrations between the ascorbic acid-deficient and control animals did not differ significantly ($P > 0.05$). The average ratio of NAD^+/NADH in the ascorbic acid-deficient animals was 0.94 and was significantly less ($P < 0.05$) than the average ratio of 1.39 obtained for the controls. The decrease in ratio of NAD^+/

TABLE 2

NAD+ and NADH concentrations in adrenal glands of male guinea pigs

Animals	Adrenal ascorbic acid	Coenzyme			Ratio, NAD+/ NADH
		NAD+	NADH	Total	
	mg/100 g fresh tissue	*moles/μg fresh tissue* × 10^{-13}			
		Ascorbic acid deficient (group 1)			
1	4.6	2.94	3.11	6.05	0.95
2	4.9	4.31	5.59	9.90	0.77
3	5.7	3.92	3.92	7.84	1.00
4	6.9	4.89	4.70	9.59	1.04
5	8.1	3.23	3.42	6.65	0.94
Avg	6.0	3.86	4.15	8.01	0.94
		Pair-fed controls (group 2)			
1	53.0	3.85	2.99	6.84	1.29
2	77.8	2.98	2.68	5.66	1.11
3	89.0	3.66	2.88	6.54	1.27
4	111.5	5.87	4.58	10.45	1.28
5	143.1	6.33	3.16	9.49	2.00
Avg	94.9	4.54	3.26	7.80	1.39

TABLE 3

NAD+ and NADH concentrations in adrenal glands of pregnant guinea pigs

Animals	Coenzyme			Ratio, NAD+/ NADH
	NAD+	NADH	Total	
	moles/μg fresh tissue × 10^{-13}			
	Ascorbic acid deficient (group 1)			
1	2.20	2.75	4.95	0.80
2	3.48	5.06	8.54	0.69
Avg	2.84	3.91	6.75	0.75
	Pair-fed controls (group 2)			
1	3.63	3.17	6.80	1.15
2	3.10	2.67	5.77	1.16
3	3.39	2.85	6.24	1.19
Avg	3.37	2.90	6.27	1.17

NADH in the ascorbic acid-deficient group is attributed to a lower NAD+ and higher NADH concentration than observed in the control animals.

The results of determinations from a few pregnant animals on the ascorbic acid-deficient diet for 15 days and their pair-fed controls show a similar trend (table 3). Visible signs of scurvy were not evident in the pregnant female. The average total NAD+ plus NADH in the ascorbic acid-deficient and pair-fed control animals was 6.75 and 6.27 × 10^{-13} moles/μg of fresh tissue, respectively. The average ratio of NAD+/NADH was 1.17 in the control animals and 0.75 in the ascorbic acid-deficient animals. In these animals also, the NAD+ concentration was lower and the NADH concentration was higher than was noted in the control animals.

NAD+ and NADH concentrations in the maternal placenta. Physical measurements of the pregnant animals used in this section of the study are presented in table 4. Because the placenta assumes an endocrine function in the second trimester of pregnancy, it was important to know the day of gestation (18). At best, the date of conception can be ascertained only to ± 1 day because of variation in time between copulation and conception. The weights and lengths of the fetuses were compared with those reported by Harman and Dobrovolny (19) for 35-day-old fetuses as an

TABLE 4

Physical measurements of pregnant animals used for coenzyme determinations

Animals	Measurements					
	No. of fetuses	Avg wt of fetuses	Avg length of fetuses	Avg wt of maternal placenta	Avg wt of fetal placenta	Wt gain of maternal organism [1]
		g	*cm*	*g*	*g*	*g*
		Ascorbic acid deficient (group 1)				
1	5	3.66	3.75	0.89	1.06	88
2	4	3.80	3.89	0.93	1.09	63
3	5	3.11	3.20	1.11	0.87	33
4	2	6.38	4.40	1.02	1.75	72
Avg	4.0	4.24	3.81	0.99	1.19	64
		Pair-fed controls (group 2)				
1	5	5.16	3.80	1.36	1.80	83
2	3	5.18	4.10	1.18	1.71	51
3	3	3.89	3.85	1.26	1.10	61
4	3	3.20	3.45	0.97	1.07	41
Avg	3.5	4.36	3.80	1.19	1.42	59

[1] From day 20 to day 35 of gestation.

TABLE 5

NAD^+ and NADH concentrations in the maternal placenta of guinea pigs

Animals	Ascorbic acid		Coenzyme [1]			Ratio, NAD^+/ NADH
	Adrenal	Maternal placenta	NAD^+	NADH	Total	
	mg/100 g fresh tissue		*moles/μg fresh tissue* $\times 10^{-13}$			
			Ascorbic acid deficient (group 1)			
1	11.80	1.68	0.97	0.43	1.40	2.26
2	13.82	1.90	0.95	0.43	1.38	2.21
3	14.88	1.68	1.24	0.67	1.91	1.85
4	27.23	3.37	0.95	0.54	1.49	1.76
Avg	16.93	2.16	1.03	0.52	1.55	2.02
			Pair-fed controls (group 2)			
1	97.04	15.76	0.84	0.22	1.06	3.82
2	98.80	—	1.02	0.34	1.36	3.00
3	112.49	13.53	0.99	0.31	1.30	3.19
4	121.04	16.14	0.82	0.25	1.07	3.28
Avg	107.34	15.14	0.92	0.28	1.20	3.32

[1] All values corrected by recoveries. Recoveries ranged from 87 to 105% for NAD^+ and from 87 to 108% for NADH.

additional estimate of the day of gestation. They reported the average weight of 35-day-old fetuses as 4.74 g with a range of 3.95 to 5.32 g and an average length of 3.94 cm with a range of 3.75 to 4.12 cm. According to these data, the animals used in this study were at or near day 35 of gestation.

Average weight gain of maternal animals in both the ascorbic acid-deficient and the control groups were similar, differing by 5 g (table 4). Food consumption in the ascorbic acid-deficient group did not decrease during the experimental period and, therefore, inanition was not a factor in the results of this study.

The fetuses and maternal organism were examined for signs of deficiency after killing. Hemorrhages were consistently noted on the head and back of the fetuses in the ascorbic acid-deficient group. These varied in severity. Slight hemor-

rhages were sometimes found in the maternal placentas of the ascorbic acid-deficient group. The maternal organism, however, showed no visible signs of deficiency.

Recovery studies were carried out on each assay by adding NAD^+ and NADH to aliquots of tissue homogenates prior to incubation. Recoveries ranged from 87 to 105% for NAD^+, and 87 to 108% for NADH. Because recoveries were somewhat more variable in maternal placenta than in adrenal gland, individual values were corrected by recoveries.

The total concentration of NAD^+ and NADH in the maternal placenta of ascorbic acid-deficient animals was 1.55×10^{-13} moles/μg of fresh tissue and does not differ significantly ($P > 0.05$) from the concentration of 1.20×10^{-13} moles/μg fresh tissue found in the control animals (table 5). The difference between the NAD^+/NADH ratio in the ascorbic acid-deficient group (2.02) and the control group (3.32) is highly significant ($P < 0.001$). The average NADH concentration in the ascorbic acid-deficient group was almost twice as high as in the control group. The NAD^+ concentration was similar in both groups.

DISCUSSION

NADH-monodehydroascorbic acid-transhydrogenase is present in adrenal gland and maternal placenta of the guinea pig. The enzyme reaction in guinea pig tissues shows some similar characteristics to that reported in tissues of other species (1–3); the reaction is specific for ascorbic acid and NADH and is inhibited by *p*-chloromercuribenzoate.

The activity of the enzyme declines as ascorbic acid concentration is decreased and in this study little or no activity could be detected when the ascorbic acid concentration was lowered to 2.3×10^{-5} M. Heath and Fiddick (3) found a similar decline in activity in bovine retinal microsomes when ascorbic acid concentration was decreased. They were able to reduce the ascorbic acid concentration to 0.23 μM, however, and still retain 30% of the activity obtained with 2.3×10^{-3} M ascorbic acid. Kersten et al. (20) were also able to reduce the concentration of ascorbic acid from 10^{-3} to 10^{-6} with porcine adrenal microsomes when a partially purified protein fraction was used with added ascorbic acid oxidase.

The response of the enzyme system to varying ascorbic acid concentrations in guinea pig adrenal gland and maternal placental tissue differs. Adrenal gland activity shows greater stimulation with increased ascorbic acid concentration than does the maternal placenta. In the intact animal, the adrenal gland contains the highest concentration of ascorbic acid of any tissue and about seven times more ascorbic acid than maternal placental tissue. The difference observed in the rate of enzyme activity in vitro may be related to the in vivo concentration and function of ascorbic acid in each tissue.

Both tissues assayed in this study are steroid-producing tissues. Scorbutic guinea pigs exhibit a deranged metabolism of corticosteroids. Corticosteroids in scorbutic adrenal glands differ both qualitatively and quantitatively from those of normal adrenals (6). The precise role of ascorbic acid in steroidogenesis has not been established. Several investigations have suggested that ascorbic acid is involved in hydroxylation reactions and in the 3-β-hydroxysteroid dehydrogenase reaction (7, 21) which leads to the production of progesterone. An important hormone itself in pregnancy, progesterone is also a key intermediate in the synthesis of other corticosteroids. Koritz (7) has demonstrated that the NADH inhibition in the conversion of pregnenolone to corticosteroid is reversed by ascorbic acid. Pankov's studies (21, 22) with pig adrenal cortex homogenates have shown that NAD^+ and ascorbic acid favor the synthesis of 17-deoxycorticosteroids whereas NADH stimulates an increased production of 17-hydroxycorticosteroids. His studies suggest that the ratio of 17-hydroxy to 17 deoxycorticosteroids may be regulated by the relative concentrations of NAD^+ and NADH and the concentration of ascorbic acid. NADH-monodehydroascorbic acid-transhydrogenase activity may be significantly related to this function in the intact organism.

Adrenal gland and maternal placental tissues of ascorbic acid-deficient guinea pigs exhibit significantly depressed ratios of NAD^+/NADH. Higher concentrations of NADH and generally lower concentra-

tions of NAD^+ account for the lower ratio of $NAD^+/NADH$ observed in the ascorbic acid-deficient animals. These findings are suggestive of an impairment in the activity of NADH-monodehydroascorbic acid-transhydrogenase with progressive depletion of ascorbic acid in the tissue. These results are consistent with the in vitro observation that decreasing ascorbic acid concentrations depress the rate of NADH oxidation by the enzyme system.

Although tissue levels of ascorbic acid were reduced to 6 to 16% of control levels, acute symptoms of scurvy were not present in either the young male or pregnant animals. The results of this study support the hypothesis that an early physiological abnormality in ascorbic acid-deficient tissues is a depressed $NAD^+/NADH$ ratio. This depressed ratio could lead to inhibition of the conversion of pregnenolone to progesterone and epimerase reactions involved in the synthesis of chondroitin sulfate precursors.

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Some Relationships Between Plasma, Liver and Excreta Tocopherol in Chicks Fed Graded Levels of Alpha-Tocopheryl Acetate [1]

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ABSTRACT Upon feeding *d*-, or *dl*-α-tocopheryl acetate to chicks, at graded levels up to 2% of the diet, both plasma and liver tocopherol concentrations plateaued between dietary intakes of 667 and 3,333 mg/kg of diet when the log of the response was plotted on the log of the dietary concentration. Balance studies determined that the percentage of the tocopheryl acetate intake, which was excreted as the ester, increased sharply between the same dietary tocopherol intakes that the plasma and liver tocopherol concentrations began to plateau. At a dietary tocopherol intake of 2%, approximately 75% of the tocopheryl acetate was excreted as the intact ester. Log plasma tocopherol concentrations, when plotted on log liver tocopherol concentrations, were linear over the entire range of dietary intakes from three experiments.

Several workers have studied the response of plasma and liver tocopherol to dietary intake of tocopherol (1–7). They all observed that plasma and liver response was linear when low, graded levels of tocopherol were fed. When amounts of up to 500 mg/day of tocopherol were fed to rats, there was a linear relationship between the log of liver tocopherol and the log of dietary intake of tocopherol (2). Similarly, in studies with chicks which were fed up to 22,046 mg of *d*-α-tocopheryl acetate/kg of diet, Wiss et al. (5) observed a linear relationship between the log of liver tocopherol concentration and the log of dietary intake of tocopherol. They found, however, that the response of plasma concentration on log of tocopherol intake was linear only when intake levels exceeded 220 mg/kg of diet. The authors concluded that this was a simple mass effect with no selective characteristics for tocopherol absorption from the gut.

In our laboratory, an initial study with chicks indicated that there was a linear relationship between the logs of both liver and plasma tocopherol and the log of dietary tocopherol concentrations, but that a plateau was reached eventually when the higher dietary levels were fed. Because the results of our study did not agree with the findings of previous investigators reported above, replicate experiments were made to corroborate our initial observations. Balance studies provided additional supportive evidence.

EXPERIMENTAL

Experiment 1. One-day-old White Plymouth Rock male chicks were raised in electrically heated batteries and fed a diet (7) low in vitamin E for 15 days. They were then randomly distributed into five groups of 24 chicks each and fed diets supplemented with either zero, 20, 200, 2,000, or 20,000 mg of *dl*-α tocopheryl acetate/kg diet for 20 days. Daily feed consumption was equalized and determined by the group with the least appetite. Eight chicks were picked at random from each of the five groups for blood and liver tocopherol analyses after 5, 10 and 20 days of supplementation. The samples from each group were pooled. Balance studies were carried out as reported previously (11) on the remaining group of eight chicks, by collecting excreta on days 18 and 19 of the supplementation period. Tocopherol analyses for plasma, liver and free and esterified tocopherol in excreta were described previously (7); the esterified tocopherol in excreta was determined as listed under the heading "Feed."

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Experiments 2 and 3. These two experiments were replicated in time, one being conducted in July and the other in December. One-day-old chicks were fed the vitamin E-low basal diet for 19 days and then divided into 11 groups of six chicks each. The chicks were then fed diets that contained *d*-α-tocopheryl acetate or *dl*-α-tocopheryl acetate at levels of zero, 26.7, 133, 667, 3,333 or 20,000 mg/kg diet for 5 days. Feed consumption for the 5-day supplementation period was regulated to the amount of diet consumed by the group of chicks with the least appetite. Excreta were collected for 48 hours on days 3 and 4 of the supplementation period for balance studies, as described previously (11), except that the times of collection were at 0800, 1400 and 2000 hours of each day. Analysis of the excreta without saponification gave the free or unesterified tocopherol concentration, whereas saponification gave the total (free plus esterified) tocopherol concentration. The amount of esterified tocopherol (reported as tocopheryl acetate) was determined by difference in concentration between saponified and unsaponified excreta, correcting for the basal concentration, and multiplying by a factor of 1.0976. This factor was obtained by dividing the molecular weight of α-tocopheryl acetate by that of α-tocopherol.

RESULTS AND DISCUSSION

Experiment 1. A plot of liver tocopherol on days of supplementation (fig. 1) showed that at the 20, 200, 2,000 and 20,000 mg/kg levels of supplementation of the basal diet, the liver tocopherol concentrations decreased from day 5 to day 20 of supplementation by approximately zero, 18, 29 and 30%, respectively. It appeared that at the 2,000 and 20,000 mg/kg levels of intake, the tocopherol concentrations might have intersected had the experiment been continued several more days. This decrease in liver tocopherol at high levels of intake was similar to the decrease we observed when massive levels of vitamin A were fed to chicks for up to 20 days (8).

Brain tocopherol concentrations at the zero and four graded levels of dietary supplementation were, respectively, 2.5, 3.1, 6.7, 20.0 and 25.8 μg/g tissue, supporting the known resistance of brain composition to change readily by dietary means.

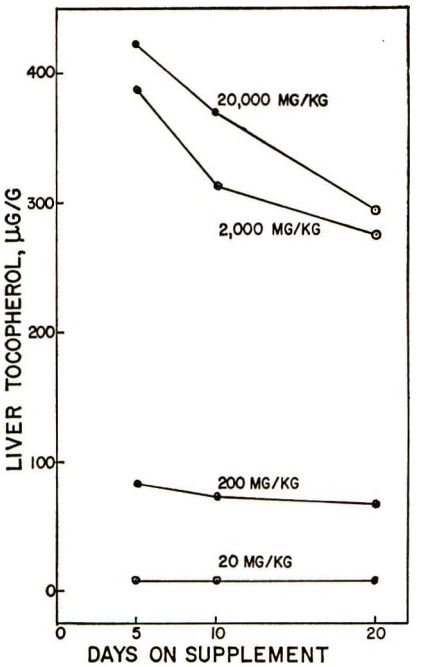

Fig. 1 The effect of feeding graded levels of *dl*-α-tocopheryl acetate, up to 2% of the diet, on 15-day-old vitamin E-depleted chicks; plotting liver tocopherol concentration on days 5, 10 and 20 of supplementation.

Experiments 2 and 3. Because these were replicate experiments, all data, after being corrected for the contribution of the basal diet, were combined and treated as one experiment. Log plasma and log liver tocopherol were plotted on log α-tocopheryl acetate intake in figure 2. This plot indicated that both the plasma and liver tocopherol concentrations plateaued when intake levels were between 667 and 3,333 mg/kg of diet. A similar plot for the data from experiment 1 (not shown) plateaued in both plasma and liver tocopherol between 200 and 2,000 mg/kg of diet.

A graph showing the amount of the tocopheryl acetate intake which was excreted as either the ester or as free tocopherol is presented in figure 3. No appreciable change took place in the percentage excreted either as free or esterified tocopherol on levels up to 667 mg/kg of diet. Excretion of the free tocopherol peaked at the 3,333 mg/kg level (2,000 mg/kg level in

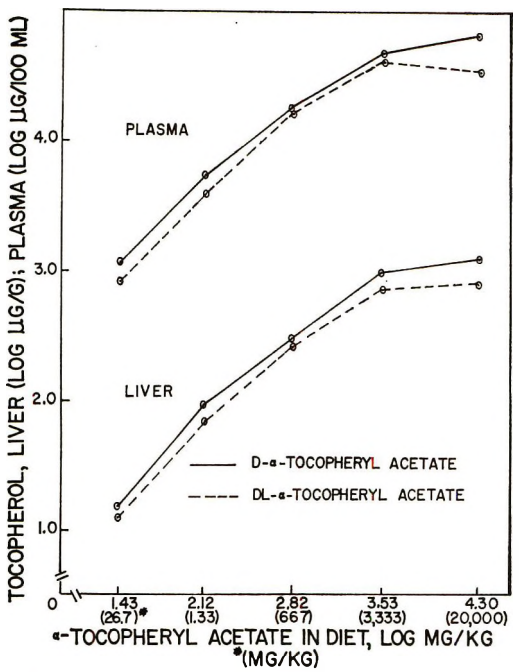

Fig. 2 The effect of feeding graded levels of *d*-, or *dl*-α-tocopheryl acetate up to 2% of the diet, and plotting the logs of plasma and liver tocopherol concentrations on the log of dietary tocopheryl acetate. Each point represents the average of two experiments and was corrected for the tocopherol concentrations obtained at the zero dietary tocopherol level. The average log plasma and log liver tocopherol concentrations at the zero level of supplementation were, respectively, 1.96 µg/100 ml and 0.26 µg/g.

exp. 1). The percentage of the tocopheryl acetate intake which was excreted as the ester increased to approximately 30% at the 3,333-mg level and to more than 70% at the 20,000-mg level (63% in exp. 1). No meaningful differences were observed in the rate of excretion between the *d*- or the *dl*-α-tocopheryl acetate which would support the work of others (9, 10) to indicate that the *l*-form is excreted more rapidly than the *d*-form. Our studies were based on the continuous feeding of tocopherol. Studies by the others were based on the administration of a single dose of radioactive tocopherol. In one chick study (10), the rate of uptake of radioactivity by the intestinal mucosa of the ligated loops was made at intervals of only up to 5 minutes, whereas in the other investigation (9), the radioactivity of the tocopherol metabolite in urine was measured for periods of up to 24 hours. Perhaps time is an important factor to consider in absorption and excretion studies.

It should be observed that the rise in tocopheryl ester excretion coincided with the plateauing of the liver and plasma tocopherol in figure 2, i.e., between the 667 and the 3,333 mg/kg diet levels of intake. It would be of interest to know the reason for the rapid increase in excretion of intact ester. Because 100% of the tocopherol in blood was reported to be transported by the β-lipoproteins (13), perhaps saturation of this complex might be the limiting factor in the amount of ester that can be hydrolyzed. Ganguly (12) reported that retinyl ester is hydrolyzed in the lumen of the small intestine by a hydrolase situated on the outer surface of the mucosal cell. Only the alcohol form crosses the cell membrane. If this same or similar mechanism operated for tocopheryl esters, then the limiting factor might be a saturation of these hydrolases by the large mass of tocopheryl ester continually passing through the intestinal tract.

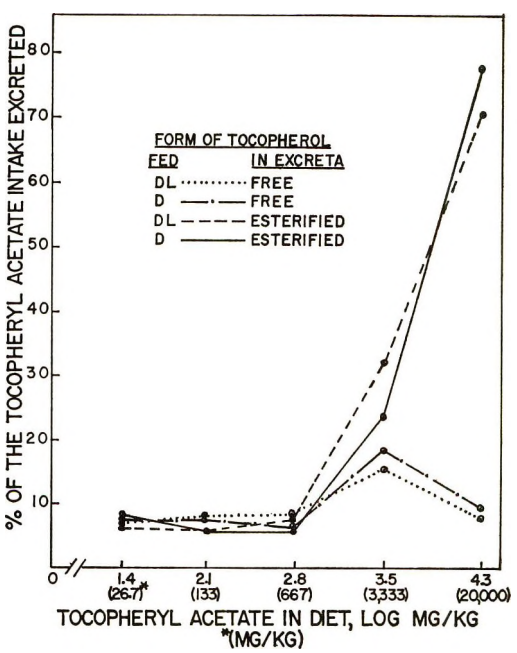

Fig. 3 A plot showing the percentage of free and esterified tocopherol excreted when the *d*-, or *dl*-α-tocopheryl acetate in the diet was fed at graded levels up to 2% of the diet.

To the authors' knowledge, no tocopheryl esters have been reported in tissue when the tocopherol was absorbed from the gut. We saponified a liver homogenate, which came from chicks whose diet was supplemented with 2% α-tocopheryl acetate, and found no change in the tocopherol level from the unsaponified sample. If esters were present, our methods of assay were not sensitive enough to detect them.

Generally, plasma tocopherol concentrations when plotted on log liver tocopherol concentrations give a linear relationship (3, 4). Our data showed a linear relationship only when the log plasma tocopherol concentration was plotted on log liver tocopherol concentration (fig. 4). It would appear that when dietary tocopherol concentrations are kept constant long enough for stabilization of the plasma and liver tocopherol, a blood sample might provide a satisfactory index of the tocopherol state of the animal, and one might also predict liver tocopherol concentrations.

ACKNOWLEDGMENTS

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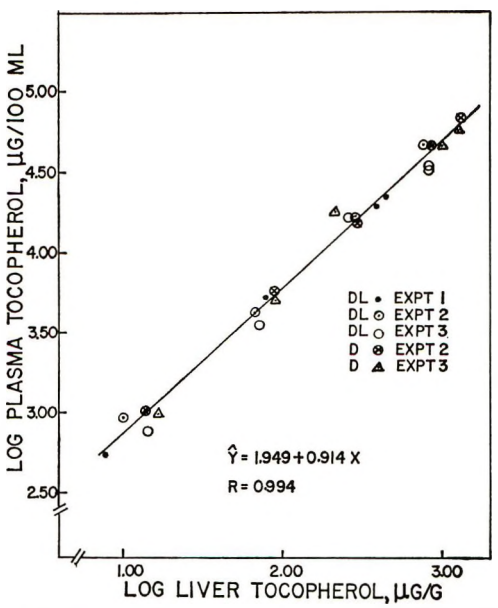

Fig. 4 A regression obtained by plotting the log of plasma tocopherol concentrations on the corresponding log of liver tocopherol concentrations from three experiments.

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Biological Activity and Excretion of the Riboflavin Analogues, 6,7-Dimethyl-9-(ω-Carboxyalkyl)-isoalloxazines in Rats [1]

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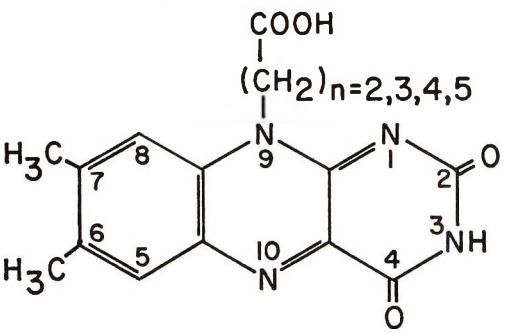
ABSTRACT A series of 9-ω-carboxyalkyl flavins with 3 to 6 carbon chain lengths are shown to weakly antagonize the utilization of suboptimal amounts of riboflavin in growing rats. This effect is most pronounced with the 3′-carboxypropyl compound. The 5′-^{14}C-carboxypentyl flavin is readily excreted in the urine, and small and trace amounts of radioactivity appear in feces and CO_2, respectively. The minor breakdown of the side chain of this analogue by intestinal microflora is decreased by succinylsulfathiazole.

Numerous analogues of riboflavin have been synthesized and tested to elucidate certain requisites of molecular structure for biological activity (1). A terminal hydroxymethyl of the side chain in position 9 of the flavin is one necessary feature, for this must be phosphorylated in a reaction catalyzed by flavokinase (2). The presence of secondary hydroxyl functions in the side chain, however, is also required for conversion of the vitamin to functional coenzyme forms, as 6,7-dimethyl-9-(ω-hydroxyalkyl) isoalloxazines are not phosphorylated (3) and are somewhat antagonistic, at high levels, to the utilization of riboflavin in *Lactobacillus casei* (4); at physiological levels they can spare normally suboptimal amounts of the vitamin in growing rats (5). Hence, more complete delineation of the relationship between structure of the side chain and biological activity of riboflavin analogues was needed. In particular, it was of interest to bioassay flavins with ω-carboxyalkyl side-chains to see if they, like the ω-hydroxyalkyl compounds, exert any riboflavin-sparing action in the rat.

The present investigation was made to assess the effects of a series of 6,7-dimethyl - 9 - (ω - carboxyalkyl)isoalloxazines with 3 to 6 carbon chain lengths on the growth of normal and riboflavin-deficient rats. Also, the loss of radioactivity from such animals with and without ingestion of a sulfa drug was measured following injection of one such flavin, the 5′-^{14}C-carboxypentyl analogue.

EXPERIMENTAL

Materials. Riboflavin [2] and sodium ^{14}C-cyanide [3] were obtained from commercial sources. The 9-ω-carboxyalkyl flavins were synthesized via the ω-cyanoalkyl derivatives as recently described (6). The 5′-^{14}C-



carboxypentyl analogue was prepared in the same way but with Na^{14}CN in synthesis of the nitrile. High purity of all flavins was ascertained by chromatography on thin-layer plates in *n*–butanol–acetic acid–water (2:1:1, v/v/v) and *n*–butanol–2 M NH_4OH–ethanol (3:1:1, v/v/v) followed by examination of the fluorescent compounds under an ultraviolet lamp. The ^{14}C-flavin was also shown to be radiochemically pure by paper chromatography and

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[1] Supported by Public Health Service Research Grant no. AM-04585 from the National Institute of Arthritis and Metabolic Diseases and by funds from the State University of New York.
[2] Purchased from the Sigma Chemical Company, St. Louis, Mo.
[3] Purchased from International Chemical and Nuclear Corporation, Burbank, Calif.

measurement of radioactivity with a radiochromatogram strip scanner.

Methods. Young male rats of the Sprague-Dawley strain,[4] weighing 50 to 60 g, were divided into groups of six animals and fed ad libitum a riboflavin-deficient test diet,[5] or the test diet to which varying levels of riboflavin, analogue, or both had been added. An additional 1% succinylsulfathiazole was added for certain experiments in which excretion of ^{14}C-flavin was measured. Animal weight was recorded before and on alternate days during the experimental period, and growth response is indicated by the average weight gain in grams. Rats which were used for determination of excretion of radioactivity were injected intraperitoneally with 1 ml isotonic saline containing 10 μg (0.5 μCi) of 5′-^{14}C-carboxypentyl flavin.

Animals were kept for 24 hours in metabolism cages for the collection of urine, feces and CO_2. Urine that was excreted was collected in flasks with 1 ml acetic acid as a preservative, and combined with urine retained in the bladder. The latter was obtained by momentarily covering the mouth and nose of the animal with ether-moistened cotton. The combined solutions were filtered and diluted to the nearest conveniently measured volume. After the animals were killed, feces and large intestinal contents, obtained by squeezing out the latter, were combined; they were autoclaved in 10 to 15 volumes of 0.1 N HCl for 15 minutes at 120°, filtered and diluted. Exhaled CO_2 was absorbed in 300 ml of 2.5 N NaOH. Aliquots of all samples were pipetted into 10 ml of Bray's solution (7), and radioactivity was determined in a liquid scintillation spectrometer.[6]

RESULTS

The rates of weight gain in rats which received 20 μg of either riboflavin or 9-ω-carboxyalkyl flavins per 15 g diet are illustrated in figure 1. The very poor growth responses obtained with the analogues were in the same range as that with the riboflavin-deficient diet alone. Hence, none of the analogues can replace the natural vitamin for growth in the rat. The rates of weight gain in rats receiving a limiting amount of riboflavin (5 μg/15 g), or this amount of the vitamin plus analogues

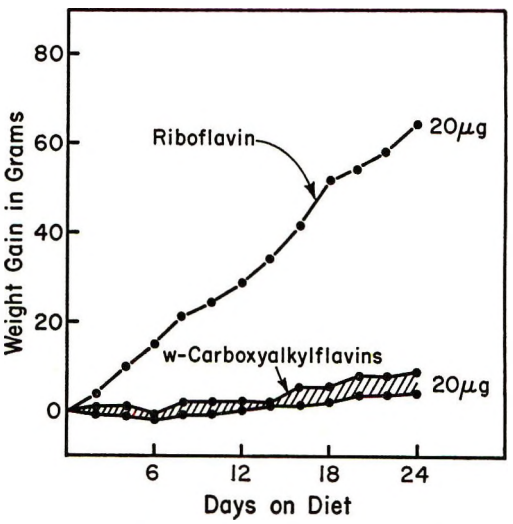

Fig. 1 Rates of weight gain in rats which received 20 μg of either riboflavin or ω-carboxyalkyl flavin per 15 g of diet. The four analogues tested had 3′-carboxypropyl, 4′-carboxybutyl, 5′-carboxypentyl, and 6′-carboxyhexyl side-chains.

(20 μg/15 g), are shown in figure 2. Some growth depression appears to be caused by the presence of all ω-carboxyalkyl flavins, but a marked effect is due to only the shortest chain 3′-carboxypropyl compound which suppressed growth almost to the level of no riboflavin. As shown by the data in figure 3, only a very mild antagonism of riboflavin can be seen with 20 μg/15 g diet of such an analogue as 5′-carboxypentyl flavin at moderately suboptimal (10 μg/15 g), as well as low (5 μg/15 g), levels of riboflavin. Even a 100-μg level of the 5′-carboxypentyl analogue caused only a modest inhibition of growth obtained on 10 μg riboflavin/15 g diet (data not shown).

The excretion and expiration of ^{14}C-activity for a 24-hour period after injection of 5′-^{14}C-carboxypentyl flavin, in normal and riboflavin-deficient rats with and without succinylsulfathiazole in the diet, is

[4] Holtzman Animal Breeders, Madison, Wis.

[5] This diet was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio and contains the following: (in percent) vitamin-free casein, 18; vegetable oil, 10; USP salt mixture no. 2, 4; sucrose and vitamins, 68. Vitamins are the following per 45.4 kg of diet: vitamin A, 900,000 units; vitamin D, 100,000 units; α-tocopherol, 5 g; ascorbic acid, 45 g; inositol, 5 g; choline chloride, 75 g; menadione, 2.25 g; *p*-aminobenzoic acid, 5 g; niacin, 4.5 g; pyridoxine·HCl, 1 g; thiamine·HCl, 1 g; calcium pantothenate, 3 g; biotin, 20 mg; folic acid, 90 mg; and vitamin B_{12}, 1.35 mg.

[6] Packard Instrument Company, Downers Grove, Ill.

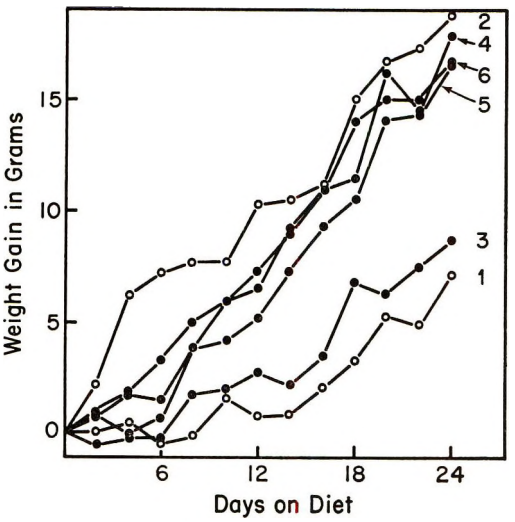

Fig. 2 Rates of weight gain in rats which received 5 μg riboflavin with and without 20 μg ω-carboxyalkyl flavin/15 g diet. Numbers for curves refer to: (1) no flavin; (2) 5 μg riboflavin; (3), (4), (5) and (6) 5 μg riboflavin plus 20 μg 3′-carboxypropyl, 4′-carboxybutyl, 5′-carboxypentyl, or 6′-carboxyhexyl flavin, respectively.

indicated by the data in table 1. Approximately half of the administered radioactivity was excreted over 24 hours, which is at least as great as a comparable dose of 2-^{14}C-riboflavin (8). Most of this was recovered in the urine. Several percent of the administered ^{14}C was observed in the feces plus large intestinal contents and is significantly decreased by the sulfa drug in both normal and deficient animals. Also, the small amount of $^{14}CO_2$ produced from catabolism of the 5′-^{14}C-carboxypentyl flavin is decreased in both normal and deficient animals by succinylsulfathiazole. This antibacterial drug appears to act by decreasing intestinal microorganisms capable of degrading the side chain of the flavin. No $^{14}CO_2$ was evolved from ^{14}C-flavin incubated with homogenates of liver or intestine of normal rats.

DISCUSSION

Quite unlike the 9-ω-hydroxyalkyl analogues of riboflavin which spare the amount of riboflavin normally required for optimal growth of young, male rats (5), a weak antagonism toward the vitamin is exhibited by its 9-ω-carboxyalkyl analogues. This may be explained by some interference of these latter compounds at the flavin

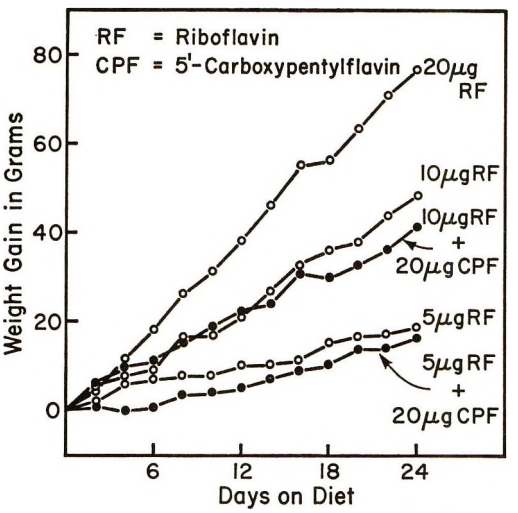

Fig. 3 Rates of weight gain in rats which received varying amounts of riboflavin with and without 20 μg 5′-carboxypentyl flavin/15 g diet.

TABLE 1

Excretion and expiration of ^{14}C-activity during 24 hours after an intraperitoneal injection of 100 μg of 5′-^{14}C-carboxypentyl flavin into rats

Dietary additions of RF [1] and SST [2]		Avg wt of three rats	Recovery of ^{14}C-activity ± SE		
Week 1	Week 2		Urine	Feces	CO_2
		g	%	%	%
—	—	57	46 ± 2.9	5.9 ± 1.4	1.8 ± 0.5
—	SST	57	40 ± 0.2	3.6 ± 0.8	0.7 ± 0.1
RF	RF	96	45 ± 2.4	7.6 ± 1.1	1.4 ± 0.2
RF	RF + SST	99	50 ± 2.5	5.6 ± 0.8	0.8 ± 0.2

[1] Riboflavin was added to the deficient diet as 20 μg/15 g.
[2] Succinylsulfathiazole was added for 1%.

coenzyme level. Because absence of hydroxyl functions in these analogues precludes their phosphorylation and conversion to analogues of the natural flavin coenzymes, and very little degradation of the side chain occurs, their action probably arises from the intact molecules. At physiological pH, these flavin acids exist as anions and thus grossly resemble the coenzyme, riboflavin 5′-phosphate. Their possible antagonism at this level of coenzyme binding and function will be sought.

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Plasma Levels of FFA, Glycerol, β-Hydroxybutyrate and Blood Glucose During the Postnatal Development of the Pig [1]

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ABSTRACT Levels of plasma free fatty acids (FFA), glycerol, β-hydroxybutyrate and blood glucose were determined in 175 sow-nursed piglets ranging in age from newborn to 9 weeks old, and in 22 newborn piglets starved up to 24 hours after birth. At birth, the concentrations of FFA and glycerol are very low. Animals starved for 6 to 24 hours from birth show a very moderate increase in FFA and unchanged or decreased concentrations of glycerol and β-hydroxybutyrate. These results are probably related to the low content of body fat in the newborn pig. During the first hours of suckling there is a significant rise in FFA and glycerol. A significant positive correlation between these parameters was found in two groups, aged 16 to 24 hours and 9 weeks. β-Hydroxybutyrate is extremely low in cord blood. A slight but significant increase is seen after birth with the peak value occurring between 8 and 12 hours. The blood glucose level is low at birth and there is a significant increase after the first nursing. The same glucose level persists throughout the first weeks of life. No correlation was found between glucose and FFA levels.

Numerous investigations of different mammals have provided considerable information about the physiological significance of the plasma free fatty acids (FFA) in energy metabolism (1). In newborn mammals (sheep, rat, man) the concentration of blood plasma FFA increases rapidly during the first hours of life (2–6). In previous studies of human newborns we also found a simultaneous increase in plasma glycerol and a subsequent rise in blood ketones and triglycerides (5). The results indicate that in the infant there is an increased mobilization and utilization of fat during the neonatal period.

Leat (7) has described the fatty acid composition of the FFA fraction of unsuckled newborn piglets and recently Swiatek et al. (8) have determined the effect of fasting on changes in plasma FFA in pigs from birth to 3 weeks of age. No systematic study has been performed concerning the postnatal changes of plasma FFA, glycerol, ketones and blood glucose in the pig. To use the pig as an experimental model for fat metabolism in pediatric research it was necessary to establish the interrelationship between these biochemical parameters and their normal pattern in the newborn and developing pig.

MATERIAL AND METHODS

A total of 197 piglets from 34 litters was obtained from 23 Swedish Landrace sows. The piglets were divided into the following age groups: newborns, 2 to 6 hours, 8 to 12 hours, 16 to 24 hours, and 48 to 72 hours; 4 to 8 days and 9 to 18 days; and 4, 5, and 9 weeks. With the exception of 14 piglets obtained from different litters and 8 from the same litter which were fasted from birth up to 24 hours, the pigs nursed on their sows ad libitum. At 14 days of age the animals were allowed free access to water and a feed mixture consisting of 75% oats and barley (1:1) and 25% of a special feed [3] for weaning piglets. The piglets were weaned after 8 weeks of age. All animals were given 150 mg iron

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[2] Requests for reprints should be addressed to Bengt Persson, M.D., Kronprinsessan Lovisas Barnsjukhus, Polhemsgaten 30, 112 30 Stockholm, Sweden.

[3] A special feed for weaning piglets, Trind, manufactured by AB Lactamin, Stockholm. Composition: 52% crude protein, 37% of which is fish raw protein, 49% digestible crude protein, 6% crude fat, 1% crude fiber and 7.5% water. Concentration of calcium was 48 and phosphorus 21 g/kg. Metabolizable energy content 3.3 Mcal/kg. The feed contained at its lowest 45 mg vitamin A, 0.375 mg vitamin D and 200 mg vitamin E/kg. It also contained trace elements, antioxidants and 135 mg antibiotics (oleandomycin)/kg.

dextrose preparation by intramuscular injection on day 3 of life.

During lactation the sows had free access to water and were given a mixture of 90% oats and barley (1:1) and 10% of a special feed [4] for pigs. The content of the mixture was 2.85 Mcal/*kg* of metabolizable energy and the level of crude protein was 119 g/kg. During the first weeks of lactation the sows were given 2.5 to 3 kg of the mixture per day.

During the study the piglets were kept in a special pen, which allowed ambient temperature to be maintained at an optimal level for each age group. A Philip Infraflex Type TS 250 heat source was used. For the first 24 hours after birth the pigs were kept at 34°. Thereafter, the temperature was lowered 1° every 48 hours up to 2 weeks of age. The older animals were kept at 23°.

Blood collection. At birth blood samples were obtained from the umbilical cord; thereafter up to 18 days of age, blood was drawn from an indwelling catheter in the external jugular vein. The catheter allowed the animal complete freedom to move. Prior to sampling, the catheter was flushed with saline. No heparin was used. Blood (1 to 2 ml) was drawn 2 to 3 hours after the pigs were removed from the sows. Pigs older than 18 days were bled by a blind puncture of the superior vena cava according to Carle and Dewhirst (9). The blood was immediately transferred to heparinized tubes and portions were deproteinized with barium hydroxide and zinc sulfate for later glucose analysis; the remainder was centrifuged at 4° and the plasma was stored at − 20° for later analysis of FFA, glycerol and β-hydroxybutyrate.

Chemical methods. All analyses were done in duplicate. Free fatty acids were determined by the colorimetric micromethod of Laurell and Tibbling (10). The error of the method for 53 duplicate analyses was ± 4.5% at a mean value of 0.341 mmole FFA/liter. Glycerol was determined by the enzymatic fluorometric micromethod according to Laurell and Tibbling (11). The methodological error obtained by 75 duplicate analyses was ± 1.5% at a mean value of 0.119 mmole glycerol/liter plasma. β-Hydroxybutyrate was analyzed on deproteinized (barium hydroxide and zinc sulfate) plasma filtrates using an enzymatic microfluorometric method according to Persson.[5] The error of the method for 74 duplicate determinations of β-hydroxybutyrate was ± 5.3% at a mean value of 0.023 mmole/liter plasma. For determination of blood glucose the enzymatic method of Marks was used (12). The analytical error was ± 1.55% at a mean value of 101 mg glucose/100 ml blood as calculated by 58 duplicate determinations. Differences between group means for blood parameters were analyzed according to Student's *t* test. The values reported are not adjusted for possible changes in plasma volume.

RESULTS

Fed animals. The mean values and standard deviations for FFA, glycerol, glucose and β-hydroxybutyrate are given in table 1. At birth, plasma FFA are extremely low but increase steadily for the first 12 hours. The mean FFA at 2 to 6 and 8 to 12 hours are significantly ($P < 0.001$) higher than those at birth. Between 12 and 24 hours of age there is a decrease in the level of FFA, but the values at 16 to 24 hours are still significantly higher ($P < 0.001$) than those in cord blood. After 16 to 24 hours FFA levels rise until 4 weeks of age. A significant ($P < 0.001$) decrease in FFA levels occurs between 4 and 5 weeks of age and between 5 and 9 weeks of age ($P < 0.01$).

Changes in glycerol concentration parallel those of plasma FFA. The mean glycerol level is significantly increased during the first 8 to 12 hours ($P < 0.001$) as compared with that of cord plasma.

A significant decrease in glycerol concentration occurs between 8 and 12 hours and 16 and 24 hours ($P < 0.01$). After 24 hours glycerol levels rise significantly ($P < 0.01$) until 4 weeks of age. There is a significant drop in glycerol level between 4 and 9 weeks of age. A significant ($P < 0.05$) correlation between FFA and gly-

[4] Suggex manufactured by AB Lactamin, Stockholm. Composition: 10% dried skim milk, 40% fish meal (70 to 74% crude protein), 26% meat meal (minimum 60% raw protein), 10% wheat bran, 8% ground limestone, 5.5% sodium chloride and trace elements and 0.5% vitamins. The content of metabolizable energy is 2.76 Mcal and the crude protein level 450 g/kg.

[5] Persson, B., manuscript in preparation.

TABLE 1

Changes in plasma FFA, glycerol, β-hydroxybutyrate and blood glucose in pigs from birth to 9 weeks of age

Parameter	FFA	Glycerol	β-Hydroxybutyrate	Glucose
	mmole/liter	*mmole/liter*	*mmole/liter*	*mg/100 ml*
Age group				
Cord	0.086 ± 0.041(38) [1]	0.052 ± 0.020(29)	0.013 ± 0.010(28)	49.9 ± 16.8(25)
2–6 hr	0.250 ± 0.18 (9)	0.143(2)	0.035(2)	91.5(2)
8–12 hr	0.290 ± 0.135(19)	0.127 ± 0.095(11)	0.166 ± 0.099(9)	99.6 ± 29.3(12)
16–24 hr	0.179 ± 0.059(17)	0.070 ± 0.038(19)	0.058 ± 0.033(13)	86.0 ± 16.9(14)
48–72 hr	0.249 ± 0.075(10)	0.086 ± 0.022(5)	0.026 ± 0.005(8)	87.3 ± 15.0(5)
4–8 days	0.296 ± 0.090(31)	0.119 ± 0.047(29)	0.038 ± 0.029(11)	81.4 ± 8.9(29)
9–18 days	0.350 ± 0.127(8)	0.120 ± 0.033(7)	0.054 ± 0.016(14)	96.8 ± 23.5(7)
4 weeks	0.450 ± 0.177(34)	0.179 ± 0.045(40)	0.022 ± 0.004(40)	102.5 ± 11.3(40)
5 weeks	0.190 ± 0.103(17)	0.071 ± 0.024(18)	0.020 ± 0.004(18)	98.2 ± 18.2(16)
9 weeks	0.088 ± 0.009(10)	0.032 ± 0.009(10)	0.027 ± 0.003(10)	109.4 ± 7.1(10)

[1] Results are given as the mean ± SD with figures in parentheses denoting number of animals.

TABLE 2

Individual values and mean values ± SD for plasma FFA, glycerol and blood glucose in 14 piglets fasted for 2 to 16 hours from birth

Age	FFA	Glycerol	Glucose
hours	*mmole/liter*	*mmole/liter*	*mg/100 ml*
2	0.09	0.079	52
2	0.10	0.091	121
2	0.13	0.081	65
2	0.11	0.043	79
2	0.15	0.059	64
3	0.10	0.035	26
3	0.09	0.046	55
3	0.09	—	—
7	0.10	0.056	62
12	0.08	—	—
12	0.15	—	76
12	0.10	—	148
12	0.14	—	86
16	0.14	0.044	36
Mean ± SD	0.11 ± 0.024	0.059 ± 0.019	72.5 ± 34

cerol is found in age groups 16 to 24 hours ($r = 0.46$), and 9 weeks ($r = 0.62$). At birth, blood glucose level is low but rises significantly ($P < 0.001$) during the following hours. During the remaining part of week 1 there are only small changes in the concentration of blood glucose. There is no correlation between FFA and glucose in any of the age groups. At birth the concentration of β-hydroxybutyrate is extremely low. Thereafter, the level is significantly ($P < 0.001$) higher in all age groups with the peak value at 8 to 12 hours.

Fasted animals. Individual and mean values for FFA, glycerol and glucose in pigs starved for 2 to 16 hours after birth are given in table 2. The mean FFA level is significantly ($P < 0.05$) higher than in cord blood. There is no significant difference in glycerol concentration but there is a significantly ($P < 0.001$) higher blood glucose level. The mean values and standard deviations for FFA, glycerol, β-hydroxybutyrate and glucose in eight littermates starved up to 24 hours are given in table 3. There is no significant difference between the FFA level at birth and 6 hours of age. A significant ($P < 0.001$) increase in FFA occurs between 6 and 24 hours. There is a significant drop in glycerol concentration during the first 6 hours ($P < 0.01$) but no significant change between 6

TABLE 3

Effect of starvation on plasma FFA, glycerol, β-hydroxybutyrate and blood glucose in newborn pigs

Parameter	FFA	Glycerol	β-Hydroxybutyrate	Glucose
	mmole/liter	*mmole/liter*	*mmole/liter*	*mg/100 ml*
Age group				
Cord	0.093 ± 0.017 [1]	0.040 ± 0.018	0.0160 ± 0.005	39.6 ± 8.7
6 hr	0.110 ± 0.024	0.013 ± 0.016	0.0046 ± 0.003	67.3 ± 18.3
24 hr	0.180 ± 0.034	0.018 ± 0.010	0.0095 ± 0.003	51.3 ± 9.6

[1] Results are given as the mean ± SD (n = 8).

and 24 hours. The blood glucose level rises significantly ($P < 0.01$) during the first 6 hours. Thereafter, a significant decrease occurs ($P < 0.05$). The β-hydroxybutyrate concentration decreases significantly ($P < 0.001$) during the first 6 hours. Between 6 and 24 hours there is a significant ($P < 0.01$) increase in β-hydroxybutyrate.

DISCUSSION

In cord blood the levels of all parameters studied are very low. The finding of low blood glucose concentration is in agreement with some studies (8, 13), but in contrast to others (14, 15). Discrepancies in reported results may be due to methodological differences. The low level of FFA in cord plasma corresponds to the results recently published by Swiatek et al. (8) and with the findings in other species studied (2, 4–6).

Within the first 2 hours of life, in unfed lambs and human infants, there is a very marked rise in plasma FFA (2, 5, 6). Studies with ^{14}C-labeled palmitate have shown that in the lamb this increase is the result of increased lipid mobilization (16). In infants the simultaneous rise in plasma glycerol concentration, which is significantly correlated to that of FFA, indicates enhanced mobilization of endogenous triglycerides (5, 6).

In contrast, the newborn pig shows a very moderate rise in FFA during starvation. A statistically significant rise in FFA occurs from 6 to 24 hours of starvation. This increase in FFA level is only twofold as compared with the sixfold increase found in newborn infants (5). A similar response to starvation has recently been reported in the pig by Swiatek et al. (8). They found, however, a significant increase in FFA level already within the first 6 hours of fasting. During fasting the concentration of plasma glycerol and β-hydroxybutyrate are low compared with human infants (5). This could indicate that the total amount of lipid mobilized during fasting is small. This finding is in good agreement with the fact that piglets lack significant adipose tissue stores at birth (17) and also that the unfed pig is very susceptible to starvation (8, 13, 18). In suckling pigs the plasma level of FFA and glycerol rises rapidly after birth. This change in FFA and glycerol becomes even greater when the dilution caused by the large increase in plasma volume during the first 24 hours of life, is considered (19). The great variability in all biochemical parameters measured in the early postnatal period is probably a reflection of large variation in composition (20) and intake of milk. From 24 hours to 4 weeks of life there is a progressive rise of plasma FFA, glycerol and glucose. At the same time there is an accumulation of body fat. Concurrently the animal develops an increased ability to tolerate prolonged starvation (8, 13). The plasma concentration of β-hydroxybutyrate, however, remains at a low level. Though significantly above the cord level the concentration of β-hydroxybutyrate never exceeds values reported in normal human infants, children or adults (5, 21).

The marked variation in FFA observed (in groups 4 and 5 weeks old), and the significant fall of plasma FFA and glycerol with no change in blood glucose that occurs after 4 weeks of age might be attributed to variable milk contributed to the diet of the animal at that time. At this time the pigs were allowed to nurse but also had free access to a feed mixture of lower fat content. The concentrations of FFA and glycerol at 9 weeks of age are very low. Slightly higher (22, 23) or much

higher (24, 25) FFA concentrations have been reported in pigs weighing 20 to 50 kg (corresponding to an age of approximately 9 to 18 weeks). These differences might be attributed to various factors, such as type of feeding and length of fasting preceding sampling.

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Effect of Fasting, Refeeding and Dietary Protein Level on Uric Acid and Ammonia Content of Blood, Liver and Kidney in Chickens

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ABSTRACT The effect of graded levels of dietary protein on ammonia and uric acid concentration in blood, kidney and liver of single comb White Leghorn cockerels was investigated. The concentration of uric acid in the blood plasma was increased proportionally to the increased protein level of the diet, and the highest value was obtained 2 hours after feeding. The blood ammonia concentration was also increased, but to a lesser degree than for uric acid, by feeding 15 to 40% casein diets, whereas zero to 10% casein diets did not affect the blood ammonia concentration. Fasting for an extended period caused a marked increase in plasma uric acid concentration; 72 and 240 hours after beginning the fast the plasma uric acid concentration reached approximately 10 and 40 times the initial concentration, respectively; the rise in uric acid concentration was reduced to normal within 6 hours after refeeding. Ammonia and uric acid contents of both liver and kidney increased gradually with the increase of dietary protein level. Kidney ammonia content per unit weight was twice the liver ammonia content, whereas total ammonia content was equal in liver and kidney. On the other hand, uric acid contents of liver and kidney per unit weight were not significantly different, whereas total uric acid content of liver was twice that of kidney.

The major urinary nitrogenous components of birds and mammals are different. Folin (1) reported that urea excretion in mammals was extremely high and variable, and that rather uniform daily amounts of ammonia, total creatinine and uric acid were excreted. In birds, however, Tasaki and Okumura (2) as well as other workers observed that the more variable urinary nitrogenous constituents were uric acid and ammonia, whereas urea and total creatinine were excreted in relatively constant amounts.

Because uric acid is the main end-product in nitrogen metabolism in birds, uric acid content in tissues would be expected to reflect the dietary protein, nutritional state, and extent or direction of protein metabolism in the birds. Bell et al. (3) observed that plasma uric acid of chickens was influenced by sex, age, nutrition and reproductive status. Nonlaying hens showed a considerably higher plasma uric acid level than laying hens, and the level of plasma uric acid was reduced by starvation. According to Sturkie (4), such differences in plasma uric acid levels in laying and nonlaying hens reflected differences in estrogen production due to different reproductive conditions.

Sturkie (5) reported that freshly drawn blood contained a very small amount of free ammonia in both birds and mammals. Okumura and Tasaki (6) observed that birds on a normal diet excreted fairly large amounts of ammonia in urine, and they found further that a portion of the excreted ammonia played a role as regulator of the acid-base balance in the animal body, and the remainder was of endogenous origin.

The present experiment was carried out to investigate the effect of fasting, refeeding and dietary protein level on uric acid and ammonia contents of bird tissues, such as blood, liver and kidney.

MATERIALS AND METHODS

Twenty-one 5-month-old single comb White Leghorn cockerels, weighing 2 kg, were used. They were housed in individual metabolic cages and divided into seven groups, three birds to a group. The birds were fed 80 g of the experimental diet [1]

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[1] The experimental diet contained: (in percent) soybean oil, 8; mineral mixture (7), 5.63; Al silicate, 1; agar-agar, 1; cellulose (provided by Sanyo Pulp Company Ltd., Gozu, Japan), 1.5; vitamin mixture (7), 1.22; and 0, 5, 10, 15, 20, 30 or 40% casein as the sole source of protein. The remainder was made up of cornstarch.

once a day, and allowed water ad libitum. The experimental birds were trained to eat about 90% of the offered diet in 20 minutes, and the remaining 10% in the next 40 minutes. In addition to the above birds, five starved birds were used to investigate the effect of fasting and refeeding on plasma uric acid content.

The birds were fed the experimental diets for 5 days, then blood samples were taken from the wing vein at zero (just before the feeding), 1, 2, 3 and 6 hours after providing the diets. The birds used for the fasting experiment were fed the 20% casein diet for 5 days, then blood samples were taken from the wing vein at 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours after the last feeding. After the birds were fasted for 240 hours, they were refed the 20% casein diet and blood samples were taken 1, 2, 3 and 6 hours after the refeeding.

Heparin was used as an anticoagulant. Immediately after collection, the blood was placed in the annular space of a Conway no. 1 unit, and blood ammonia was determined by the method of Conway (8) with photometric means. Plasma uric acid was determined by the method of Bergman and Shabtay (9) through the absorbancy of the supernatant at 290 mμ. After the birds were fed the experimental diets for 10 days, they were decapitated 2 hours after feeding. The kidney and liver were immediately removed; they were homogenized with 4 parts of 10% ethyl alcohol to prevent coagulation according to Homma and Sato (10). Ammonia and uric acid concentrations of the homogenate were estimated by the methods of Conway (8) and Bergman and Shabtay (9), respectively.

RESULT AND DISCUSSION

The time-course of plasma uric acid concentration for various levels of dietary casein is shown in table 1. Just before feeding, namely 24 hours after the previous feeding, the plasma uric acid concentration was relatively constant even if the dietary casein level was different. After feeding the casein diets, plasma uric acid level increased sharply, and the highest value was obtained 2 hours after feeding. It was also observed that the higher the level of dietary casein, the higher the plasma uric acid concentration ($r = 0.898$ at 2 hours after feeding). After it reached the highest level, the concentration decreased gradually. When 5, 10, 15 and 20% casein diets were fed, plasma uric acid concentrations, 3 hours postfeeding, approached the level at zero time, whereas the birds fed 30 and 40% casein diets still maintained significantly higher levels of plasma uric acid.

Bell et al. (3) reported that the average concentrations of plasma uric acid of laying hens, nonlaying hens, immature hens and cockerels were 2.27, 5.40, 4.38 and 2.86 mg/100 ml plasma, respectively. The data obtained in the present experiment indicated that the plasma uric acid concentration was affected by the dietary protein level and by time after feeding. Such a high plasma uric acid concentration on high casein diets presumably indicates an increased exogenous nitrogen metabolism.

Lewis (11) used ruminants, Puchal et al. (12) baby pigs, and Addis et al. (13) human beings to show that the amounts of ingested protein influenced the level of circulating blood urea. Siller (14) reported that in chickens a high protein diet increased plasma urate. The present ex-

TABLE 1

Effect of varying levels of dietary protein on the plasma uric acid concentration (mg/100 ml plasma)

Hours after giving diet	0	1	2	3	6
Protein-free diet	5.6 ± 0.4 [1]	5.5 ± 0.4	4.8 ± 0.4	5.5 ± 0.3	5.6 ± 0.3
5% casein diet	5.5 ± 0.3	6.8 ± 0.2	7.0 ± 0.3	6.0 ± 0.4	6.5 ± 0.3
10% casein diet	5.8 ± 0.5	8.1 ± 0.8	7.5 ± 0.0	6.2 ± 0.4	6.5 ± 0.4
15% casein diet	6.4 ± 0.6	8.4 ± 0.8	10.8 ± 0.9	7.9 ± 0.3	7.5 ± 0.2
20% casein diet	6.6 ± 0.2	10.7 ± 0.2	11.2 ± 1.7	8.3 ± 0.9	8.1 ± 1.0
30% casein diet	6.4 ± 0.6	13.9 ± 3.5	14.8 ± 4.3	13.3 ± 2.7	11.2 ± 1.3
40% casein diet	7.4 ± 0.7	18.4 ± 1.9	23.0 ± 3.2	13.9 ± 1.2	12.2 ± 1.2

[1] Mean ± SE of mean.

TABLE 2

Effect of varying levels of dietary protein on the blood ammonia concentration (ammonia N mg/100 ml blood)

Hours after giving diet	0	1	2	3	6
Protein-free diet	0.31 ± 0.03 [1]	0.36 ± 0.04	0.34 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
5% casein diet	0.32 ± 0.03	0.35 ± 0.03	0.33 ± 0.03	0.34 ± 0.04	0.33 ± 0.03
10% casein diet	0.31 ± 0.02	0.33 ± 0.03	0.32 ± 0.02	0.31 ± 0.03	0.31 ± 0.02
15% casein diet	0.35 ± 0.02	0.42 ± 0.04	0.54 ± 0.06	0.51 ± 0.05	0.35 ± 0.04
20% casein diet	0.35 ± 0.03	0.47 ± 0.04	0.59 ± 0.05	0.56 ± 0.05	0.37 ± 0.04
30% casein diet	0.28 ± 0.03	0.51 ± 0.03	0.65 ± 0.05	0.57 ± 0.05	0.37 ± 0.03
40% casein diet	0.37 ± 0.03	0.50 ± 0.04	0.63 ± 0.06	0.55 ± 0.06	0.40 ± 0.04

[1] Mean ± SE of mean.

periment also showed that the quantity of absorbed nitrogen reflected the plasma uric acid in the chicken. The results obtained with blood uric acid in birds resemble those with blood urea in mammals, since mammals are ureotelic and birds are uricotelic animals. Tasaki and Okumura (2) reported that the urinary nitrogen excretion is linearly proportional to the nitrogen intake. The present experiment shows that increased protein intake results in the increase of plasma uric acid concentration. Consequently, the higher plasma uric acid concentration will result in increased excretion of nitrogen in urine.

Table 2 shows the time-course of the blood ammonia concentration for varying levels of dietary protein. Before feeding the diet, the blood ammonia nitrogen concentration was approximately 0.33 mg/100 ml. Feeding zero, 5, and 10% casein diets did not change the concentration of blood ammonia during the 6-hour experimental period. On the other hand, the blood ammonia concentration was gradually increased, but to a lesser degree than for uric acid, by feeding 15, 20, 30 and 40% casein diets, and the highest level was observed 2 hours after feeding; 6 hours after feeding, the blood ammonia concentration returned to the initial level. Harper et al. (15) reported that the blood ammonia concentration in rats was increased by the administration of amino acids or a casein hydrolysate. The present experiment showed that in birds the blood ammonia is increased by the feeding of 15, 20, 30 and 40% casein diets, but not by feeding diets which contain less than 10% casein. Barnes et al. (16) reported that the protein source has a marked effect upon the level of ammonia in the rat portal vein. The same protein (casein) was used throughout the present experiment, and proteins other than casein may produce different results.

The effect of dietary protein level on ammonia and uric acid contents of both liver and kidney is illustrated in figures 1 and 2.

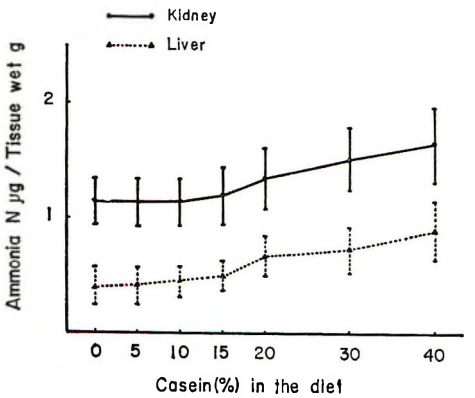

Fig. 1 The effect of varying levels of dietary protein on liver and kidney ammonia contents.

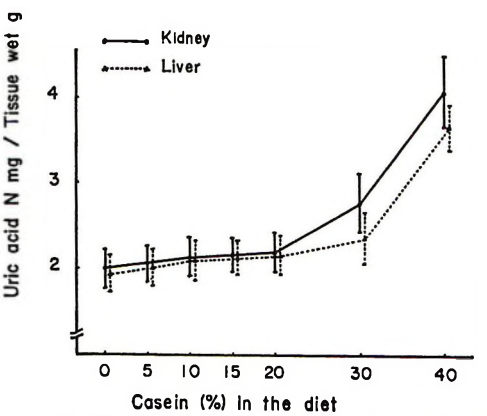

Fig. 2 The effect of varying levels of dietary protein on liver and kidney uric acid contents.

Ammonia content of both liver and kidney increased gradually with the increased dietary protein level. Although ammonia content per unit weight of kidney was approximately twice that of liver, total ammonia content was almost equal in liver and kidney, because the kidney weight (average 14.3 g) was about one-half the liver weight (average 34.2 g). Uric acid content of both liver and kidney also increased gradually with the increased dietary protein level; however, the marked increase was observed in birds fed 30 and 40% casein diets. Total liver uric acid content was approximately twice that of total kidney uric acid content, whereas no significant difference of uric acid content per unit weight was found in liver and kidney. The significance of uric acid and ammonia content in the tissues will be discussed in a separate report which considers xanthine dehydrogenase and glutaminase activities.

The effect of fasting and refeeding a 20% casein diet on the plasma uric acid concentration is shown in figure 3. Fasting for 48 hours did not significantly affect the plasma uric acid concentration, but in the next 24 hours (72 hours of fasting) the plasma uric acid concentration increased significantly, and reached as much as 10 times the initial concentration (70 ± 3 mg/100 ml plasma). When fasting was continued further, the plasma uric acid concentration increased gradually until 120 hours; then the plasma uric acid concentration increased dramatically, and 240 hours after fasting it reached approximately 40 times the initial concentration (251 ± 26 mg/100 ml plasma). The average body weight loss of the experimental birds was 110, 420 and 634 g at 24, 120 and 240 hours of fasting, respectively; two of five birds died at 216 and 240 hours of starvation.

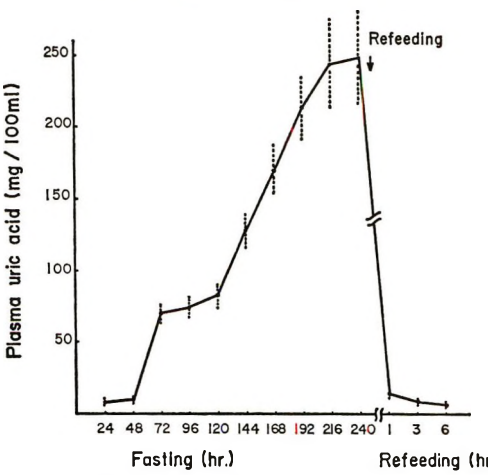

Fig. 3 The effect of fasting and refeeding a 20% casein diet on the plasma uric acid concentration. The vertical lines through each point represent standard errors of means.

After 240 hours of fasting, the surviving birds were refed a 20% casein diet. By refeeding the diet, the plasma uric acid concentration fell very quickly, and reached 14.3 mg/100 ml plasma 1 hour after refeeding; the concentration decreased gradually to the base level within 6 hours.

Bell et al. (3) reported that 36 hours of starvation depressed the plasma uric acid level of birds. Siller (14) also reported that starvation decreased the plasma uric acid level significantly. Homma and Sato (10), however, reported that fasting of birds increased the plasma uric acid concentration. The present experiment indicated that fasting for 48 hours did not affect the plasma uric acid level of birds, but fasting for 72 hours increased the plasma uric acid level significantly. Specifically, fasting for 240 hours greatly increased the plasma uric acid level. When birds were refed a 20% casein diet, the plasma uric acid level fell drastically; 6 hours after refeeding, the plasma uric acid returned to the base level observed at the time of the 24-hour fast. A high plasma uric acid concentration observed in starved birds would indicate an increased catabolism of tissue protein. This assumption would be supported by the fact that xanthine dehydrogenase activity of chick liver was enhanced by starvation (Corte and Stirpe (17)). The drastic decrease in the plasma uric acid after refeeding might be the reflection of the depressed catabolism of tissue protein.

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Studies on the Mechanism of Copper Absorption in the Chick

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ABSTRACT The mode of copper absorption from the gastrointestinal tract of chicks has been investigated. Studies with ^{64}Cu indicated that copper absorption was greater from the duodenum than from the proventriculus. No absorption of copper was observed from the ventriculus. Copper-64 present in the duodenal mucosa was found to be firmly and specifically attached to protein with a molecular weight of approximately 10,000. Zinc and cadmium, inhibitors of copper absorption, appear to act as copper antagonists by binding to, and displacing copper from, the 10,000 molecular weight duodenal protein.

Relatively little is known about the mechanism of copper and zinc absorption from the gastrointestinal tract. Most investigations in this field have been concerned with the effect of metals which are antagonistic to copper and zinc utilization (1–5). Studies by Van Campen and Scaife (5) indicate that the site of this antagonism is located in or on the intestine, suggesting a certain degree of specificity and implying the possibility of the existence of an active transport system. Whether the action of the antagonist is to block hypothetical protein binding sites or to inhibit a more complex transport system is not known.

Sahagian et al. (6), using an in vitro perfusion technique, have suggested, on the contrary, that the movement of zinc and several other trace minerals across the intestinal membrane is not mediated by an active transport mechanism, but is controlled merely by the diffusibility characteristics of the individual ions.

This paper presents the results of experiments designed to investigate the mechanism of copper absorption. The evidence gathered demonstrates the presence, in the duodenum of chicks, of copper-binding protein which may play a fundamental role in the process of copper absorption.

MATERIALS AND METHODS

Arbor Acre line 50 chicks were used throughout these studies. They were housed in conventional batteries with raised wire floors. Commercial chick starter [1] and tap water were given ad libitum. Chicks were fasted overnight prior to an experiment.

Copper-64 and cadmium-115m as their nitrates, and manganese-54 and zinc-65 as their chlorides, were used.[2] When administered orally, approximately 10 μCi of ^{65}Zn, ^{115m}Cd, and ^{54}Mn, and 40 μCi of ^{64}Cu, were given in a volume of 0.2 ml. The absolute amount varied between 1 and 30 μg. After allowing 2 hours for absorption, the chicks were killed; the organs to be studied were removed, opened when necessary, and washed with cold water. Duodenal homogenates were made in three volumes of 0.05 M potassium phosphate buffer, pH 7.2, and centrifuged at 25,000 × *g* for 60 minutes.

When administered orally as antagonistic elements, cadmium, zinc, mercury, manganese, and molybdenum were given as the sulfates. Silver was given as the nitrate.

Gel filtration was performed at 22° on Sephadex G-100 columns 2.5 cm by 41 cm, equilibrated at pH 6.8 with 0.025 M potassium phosphate buffer containing 0.05 M potassium chloride. Flow rates were adjusted to 0.5 ml/minute. The columns were standardized for molecular weight approximation using bovine serum albumin, cytochrome c and ^{64}Cu. Radioactivity in each column fraction was measured in a well scintillation counter.[3] Protein was measured by absorbance at 280 mμ.

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[1] Wayne Pullet Starter, Allied Mills, Inc., Chicago, Ill.

[2] Obtained from Nuclear Science and Engineering Corporation, Pittsburgh, Pa.

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

All dialysis experiments were performed in the pH 6.8 phosphate buffer described above.

RESULTS AND DISCUSSION

In the rat, copper is absorbed from both the stomach and the duodenum to about the same extent, whereas zinc is absorbed much more readily from the duodenum (7). Because the anatomy of the chick is different from that of the rat, it was necessary to do preliminary absorption studies on ligated segments of the chick gastrointestinal tract. Ten-day-old chicks were anesthetized with chloroform, and the proventriculus, ventriculus, duodenum, and the remaining segment of the small intestine up to the point of junction of the cecum were ligated according to the procedure of Van Campen and Mitchell (7). Forty microcuries of ^{64}Cu in 0.2 ml were injected into the ligated segment, and after allowing 2 hours for absorption, the chicks were killed; the livers were removed and the ^{64}Cu content was measured. Since the liver is a major storage area for copper, the incorporation of radioactivity into this organ was used as a measure of absorption. Table 1 presents the results of this experiment, designed to identify the gastrointestinal segment of chicks from which copper is most readily absorbed. Although significant absorption occurred from both the proventriculus and duodenum, the absorption from duodenum was about five times greater than that from the proventriculus. Attempts to measure absorption from the remaining segments of the small intestine were not feasible, for the birds died before the 2-hour absorption period was completed. The data of Van Campen and Mitchell (7) showed that absorption took place in the jejunum and the ileum of the rat, but not to the same

TABLE 1

Absorption of ^{64}Cu from ligated sections of the gastrointestinal tract

Segment	
	cpm per liver [1]
Proventriculus	912 ± 453
Ventriculus	15 ± 8
Duodenum	4,846 ± 1,668

[1] Each value is the average from four chicks ± SE of the mean.

TABLE 2

Distribution of orally administered ^{64}Cu, ^{65}Zn, ^{115m}Cd and ^{54}Mn [1]

Segment	^{64}Cu [2]		^{64}Cu + EDTA [2]		^{65}Zn [2]		^{115m}Cd [2]		^{54}Mn [2]	
		% [3]		% [3]		% [3]		% [3]		% [3]
Proventriculus	6,934 ± 2,331	6	1,211 ± 353	2	3,788 ± 1,110	4	23 ± 27	1	665 ± 277	1
Ventriculus	125,685 ± 44,393	87	31,962 ± 12,460	68	11,720 ± 1,560	12	366 ± 103	5	5,793 ± 2,027	6
Duodenum	6,912 ± 2,239	5	6,677 ± 2,279	14	57,985 ± 18,053	60	5,875 ± 1,989	93	95,676 ± 34,496	89
Liver	4,372 ± 1,551	3	7,417 ± 2,358	16	22,900 ± 7,245	24	26 ± 23	1	4,783 ± 1,664	4

[1] Each value is the average from four chicks ± SE of the mean.
[2] Counts per minute per tissue.
[3] Percentage of recovered dose.

extent as from the duodenum. We concluded from our results that in the chick, copper is absorbed to a much greater degree from the duodenum than from the stomach, and therefore chose the duodenum in the search for possible metal-binding proteins which might play a role in the absorption process.

Although there was no absorption from the ventriculus, this organ had an extraordinary ability to bind copper. Table 2 shows the distribution of orally administered radioisotopes 2 hours after administration. Roughly 90% of the recovered dose of ^{64}Cu was retained by the ventriculus, and dissection showed this to be located on the interior surface, associated with the tough, horny layer of the lining. Much smaller amounts of the other isotopes were bound to this layer. The significance of this ability to bind minerals, particularly copper, is not apparent. In experiments involving oral doses of copper, however, it must be kept in mind that this trapping of copper by the ventriculus can reduce the effective dose available for absorption. The presence in the diet of various factors, such as natural chelating agents, probably affects the utilization of this "trapped" copper. This is illustrated by the effects seen when 1 mg EDTA is given orally at the same time as ^{64}Cu (table 2, column 2). In this situation, much less ^{64}Cu is bound to the ventricular lining than in the absence of EDTA, and the relative amount recovered in the liver is increased fivefold.

Although copper was found to be present in the duodenal mucosa, this did not necessarily mean that it was protein bound. Experiments were conducted to test the protein-binding hypothesis. Dialysis overnight against 100 volumes of buffer removed less than 5% of the ^{64}Cu from pooled duodenal homogenates from chicks given ^{64}Cu orally, and addition of 5 ml of 10% trichloroacetic acid (TCA) as a protein precipitant to 5 ml of the duodenal homogenates precipitated more than 90% of the radioactive copper. Ammonium sulfate fractionation of duodenal homogenates from chicks given an oral dose of ^{64}Cu yielded the distribution shown in table 3. The major portion of the ^{64}Cu precipitated at an ammonium sulfate concentration of 45 to 80% saturation; only 4% remained in solution as nonprotein-bound copper.

These experiments indicated that ^{64}Cu was bound to one or more proteins in the duodenum. To test this more stringently, and to characterize more fully the copper-binding protein(s), a 5-ml sample of duodenal homogenate from chicks given ^{64}Cu orally was applied to a Sephadex G-100 column. Figure 1 shows the elution pattern of ^{64}Cu and protein. Only one major radioactive peak was observed, with an elution volume of 160 ml. The estimated molecular weight for protein with this elution volume is approximately 10,000. Addition in vitro of 0.5 μCi of ^{64}Cu to duodenal ho-

TABLE 3

Ammonium sulfate fractionation of ^{64}Cu-labeled duodenal homogenate

Fraction	^{64}Cu
	%
Homogenate (supernatant 25,000 × *g*)	100
0 to 45% saturated $(NH_4)_2SO_4$ precipitate	14
0 to 45% saturated $(NH_4)_2SO_4$ supernatant	86
45 to 80% saturated $(NH_4)_2SO_4$ precipitate	82
45 to 80% saturated $(NH_4)_2SO_4$ supernatant	4

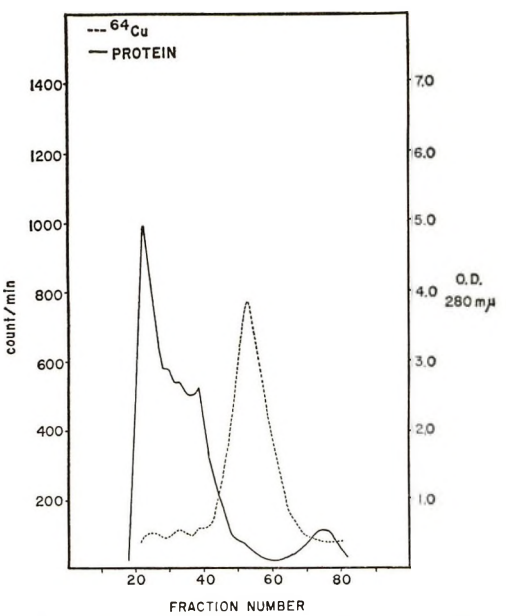

Fig. 1 Isotope and protein elution pattern from a Sephadex G-100 column of duodenal homogenate from chicks given an oral dose of ^{64}Cu. Column size was 2.5 cm by 41 cm; fraction size was 3 ml.

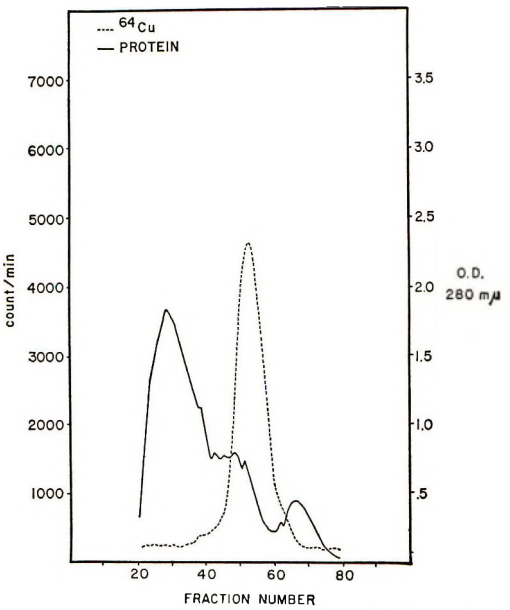

Fig. 2 Isotope and protein elution pattern from a Sephadex G-100 column of duodenal homogenates to which ^{64}Cu had been added in vitro. Column size was 2.5 cm by 41 cm; fraction size was 3 ml.

mogenates from untreated chicks gave an elution pattern from a Sephadex G-100 column identical to that of the orally administered ^{64}Cu, displaying very specific binding to the 10,000 molecular-weight protein(s) with virtually no absorption to the other proteins (fig. 2). On the other hand, ^{64}Cu chromatographed on the same column, but without protein, had an elution volume unambiguously different, 210 ml.

From these experiments it was concluded that copper was associated in the duodenum rather specifically with protein of approximate molecular weight of 10,000. The question of whether or not this copper-binding protein was involved in any way in the absorption process, however, remained unanswered. The well-known ability of certain elements to act as antagonists to copper absorption was next exploited in an attempt to answer this question. If metals antagonistic to copper absorption accomplish this antagonism by displacing copper on the copper-protein complex described above, then this should be experimentally verified by demonstrating that antagonistic elements do indeed bind to the same protein, whereas indifferent elements do not. There should also be a marked reduction in the ^{64}Cu bound to this protein in the presence of an antagonistic element. Several elements, zinc, silver, mercury, cadmium, manganese and molybdenum, some of which are known copper antagonists, were given orally 30 minutes prior to the administration of an oral dose of ^{64}Cu. In each case, 1 mg of each antagonistic element was given in 0.2 ml water that had been adjusted to pH 5 to 6. Table 4 shows the results of these experiments. The elements zinc, cadmium and silver, previously shown to have an interaction with copper metabolism (1–5), were strong inhibitors of copper absorption, whereas manganese and molybdenum had no visible effect. Mercury lowered copper absorption but not to the same extent as the other antagonists. In-

TABLE 4

Effect of orally administered metals on ^{64}Cu absorption and the amount of ^{64}Cu bound to duodenal protein

Metal	Percent of control [1]			
	Absorption [2]	P [3]	Duodenal protein-bound ^{64}Cu	P [3]
None	100	—	100	—
Manganese	101	> 0.8	80	> 0.4
Molybdenum	99	> 0.9	—	—
Mercury	61	$< 0.2; > 0.1$	101	> 0.9
Zinc	33	< 0.01	36	< 0.01
Silver	28	< 0.001	65	$< 0.2; > 0.1$
Cadmium	20	< 0.001	9	< 0.001

[1] Average of four chicks in each group.
[2] Measured by ^{64}Cu incorporation into the liver.
[3] Probability, by *t* test, that experimental values are the same as the control.

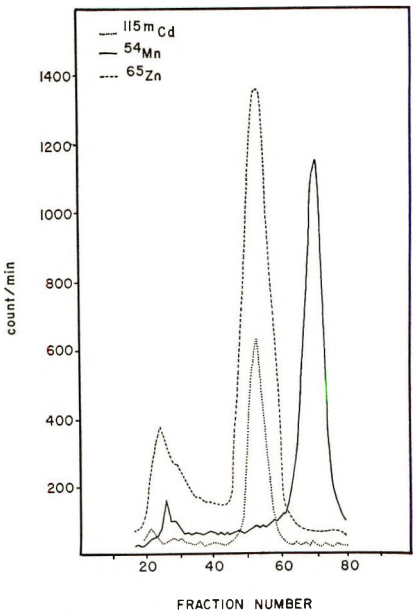

Fig. 3 Isotope elution pattern from Sephadex G-100 columns of duodenal homogenates from chicks given an oral dose of either ^{65}Zn, ^{54}Mn or ^{115m}Cd. Column size was 2.5 cm by 41 cm; fraction size was 3 ml.

TABLE 5

Dialysis of duodenal homogenates labeled with ^{64}Cu, ^{115m}Cd, ^{65}Zn and ^{54}Mn

	Undialyzed	Dialyzed	Percent removed by dialysis
	cpm	*cpm*	*cpm*
^{64}Cu	7,531	7,219	4
^{115m}Cd	1,718	1,566	9
^{65}Zn	41,097	30,707	25
^{54}Mn	4,304	954	78

corporation of ^{64}Cu into duodenal protein was also markedly reduced in the presence of zinc and cadmium. This would be expected if the antagonists were blocking copper-binding sites on the duodenal protein. A much smaller reduction of ^{64}Cu incorporation into duodenal protein was observed in the presence of silver, and there was no effect on duodenal bound ^{64}Cu in the presence of mercury, although both elements had an effect on ^{64}Cu absorption. The reason for this is not clear. Perhaps mercury and silver differ from cadmium and zinc in the mechanism by which they inhibit copper absorption.

The possibility that these antagonists were acting by absorbing to the copper-binding protein was further explored by chromatographing duodenal homogenates from chicks given oral doses of ^{65}Zn, ^{115m}Cd and ^{54}Mn on Sephadex G-100 columns. Figure 3 shows the isotope elution pattern of these elements. Both ^{115m}Cd and ^{65}Zn absorb to protein with the same elution volume as the copper-binding protein, whereas ^{54}Mn elutes much later (elution volume 210 ml), which corresponds exactly to the elution volume of free ^{54}Mn. The marked difference in the binding of ^{54}Mn to duodenal protein is also shown by the effect of dialysis overnight against 100 volumes of buffer (table 5). Manganese-54 is readily dialyzable, whereas ^{64}Cu, ^{115m}Cd and ^{65}Zn are not, again suggesting that ^{54}Mn present in the duodenum is not protein bound whereas the other elements are.

From these studies it appears that the binding of copper to duodenal protein with approximate molecular weight of 10,000 is an important step in the process of copper absorption. Cadmium and zinc inhibit copper absorption by absorbing to, and competing for, the same protein-binding sites in the duodenum required for copper. Whether these are simple binding sites or have a more complicated transport function is not known, nor can it be said with certainty that the same protein is involved in the absorption of elements other than copper. Zinc and cadmium, however, were associated with protein of the same molecular weight as copper-binding protein and perhaps are involved in a similar absorption process.

ACKNOWLEDGMENTS

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Pancreatic Enzymes in Germfree and Conventional Rats Fed Chemically Defined, Water-soluble Diet Free from Natural Substrates [1]

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ABSTRACT The effects of intestinal microflora and chemically defined, liquid diet free from natural substrates (for pancreatic enzymes), on pancreatic function and on fate of pancreatic enzymes, were studied using germfree and conventional rats fed liquid and semipurified solid diets. Histopathological examination of pancreatic sections of rats fed either diet showed that pancreatic acinar cells in all sections were normal. Serum lipase and amylase levels were normal in germfree and conventional rats fed either diet. Lipase and amylase levels in the pancreas were not affected either by germfree status or by diets. Germfree status had no effect on trypsinogen and chymotrypsinogen in the pancreas, whereas feeding of liquid diet resulted in lower activities of these enzymes in both germfree and conventional rats as compared with those fed a semipurified diet. Feeding of liquid diet and presence of microflora were associated with a lower activity of lipase in the contents of cecum and large intestine, and with a decrease in activities of amylase, trypsin and chymotrypsin in small intestine, large intestine and cecum. These results indicate that the pancreatic enzymes undergo a progressive inactivation on entering the small intestine, and subsequently, passing through the entire intestinal tract. Diets without natural substrates and microflora played an important role in inactivation of these enzymes in the intestinal tract. It is concluded that the chemically defined, liquid diet used in this study was nutritionally adequate for germfree and conventional rats as far as normalcy and function of pancreas were concerned.

Germfree animals offer a unique tool in nutritional and immunological studies. It has been difficult to take full advantage of this tool, however, because the diets for germfree animals must be sterilized, and even well-defined diets suffer changes of uncertain character and extent when sterilized by heat, radiation or chemical agents (1). Recently, however, Pleasants [2] has developed a chemically defined, water-soluble (liquid) diet containing amino acids, dextrose, ethyl linoleate, vitamins and minerals which can be sterilized by filtration without loss of chemical definition (2, 3). The nutritional adequacy of this diet has been demonstrated because it has supported reproduction of germfree mice into the fifth generation and growth of germfree rats from birth through maturity (2). These results indicate that the diet is at least qualitatively adequate for rats and mice. If this diet-animal system is to prove a useful tool in nutritional and immunological studies, it is important to determine whether the animal is physiologically "normal" in a chemical environment thus limited and defined.

Preliminary observations of Geever et al.[3] suggested that a chemically defined liquid diet (differing from that of Pleasants [4] chiefly in containing Tween 80 and in lacking chromium and selenium) produced atrophic and fibrotic changes in the acinar (exocrine) cells of the pancreas. Wiech et al. (4) demonstrated, in germfree rats fed normal stock diet, a delayed and decreased insulin secretion, suggesting

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[1] Supported specifically by Public Health Service Research Grant no. HD-00855 from the National Institutes of Child Health and Human Development; also by the University of Notre Dame.

[2] Pleasants, J. R. 1966 Development of chemically defined, water-soluble diets nutritionally adequate for germfree rats and mice. Ph.D. thesis, University of Notre Dame, Notre Dame, Indiana.

[3] Geever, E. F., F. S. Daft and S. M. Levenson 1965 Pancreatic atrophy and fibrosis in the germfree rat on a chemically defined diet. Federation Proc., 24: 246 (abstract).

[4] See footnote 2.

some effect of the germfree state either on the pancreas in general or on the endocrine pancreas in particular. The types of changes seen by Geever et al.,[5] when observed in other types of investigations, have been associated with changes in the activities of pancreatic digestive enzymes such as amylase, lipase, trypsin, and chymotrypsin (5, 6). Borgstrom et al. (7) noted an increase in the activity of trypsin in the feces of germfree rats as compared with conventional rats, when both were fed a natural-type diet. Thus, pancreatic function and its more remote effects can be influenced by both microbial status and by diet. Dietary influences, in turn, could result either from an actual deficiency or imbalance of materials such as amino acids needed for efficient enzyme production, or from lack of exogenous stimulation of enzyme production (8–14). Because the chemically defined liquid diet provides none of the natural substrates for pancreatic enzymes (proteins, fats and carbohydrates), this situation alone might lead to pancreatic changes having unexpected consequences.

A study of pancreatic enzymes in germfree rats fed liquid diets would therefore provide an indicator of pancreatic function under such conditions, and an indirect indicator of dietary adequacy. Secondly, the data would provide a well-controlled evaluation of the role of natural substrates as inducers of pancreatic enzyme production. Thirdly, the data would indicate how much the microbial flora affects enzyme levels within the pancreas and in the lumen of the intestine when animals are fed widely differing types of diet. It would thus be possible to assess the normalcy of pancreatic function in germfree rats fed a defined diet and also the factors which might account for changes from expected levels of functioning. The data would contribute to the understanding of pancreatic function, and also improve the usefulness of the germfree animal and defined diet system for other types of research in which pancreatic function could be an important variable.

MATERIALS AND METHODS

The germfree and conventional male albino rats of Lobund stock (Wistar origin) used in this investigation were genetically closely related. All rats were housed in individual screen-bottom cages and maintained as described in an earlier report (15). To wean the germfree and conventional rats directly from mother's milk to experimental diets without their consuming nonexperimental diets offered to the mothers, the procedure outlined in our previous investigation was followed (3). Germfree and conventional rats were fed ad libitum one of the following experimental diets (table 1): a Millipore-filtered, chemically defined, water-soluble diet (L-479 E9) based on amino acids, glucose and ethyl linoleate (3) and a steam-sterilized, semipurified diet (L-474 E12), based on purified casein, starch and corn oil (16). All animals were fed the experimental diets until 100 days of age.

After the experimental period, germfree and conventional rats were fasted for 3 hours with free access to water, then killed. The animals were first anesthetized with ether; blood was collected directly from the heart, and then the rats were decapitated. The intestinal tract and pancreas were excised immediately. The pancreas was freed from adhering fat and connective tissue and weighed; it was quick-frozen immediately in dry-ice and stored at $-20°$ until the next day when enzyme determinations were made. Unfrozen pancreatic glands were used for histological studies. The contents of the small intestine, large intestine and cecum were washed out separately with ice-cold 0.15 M saline into polyethylene tubes and frozen at $-20°$ until the next day.

Each pancreas was homogenized with 10 ml of ice-cold 0.15 M NaCl/g tissue in a Potter-Elvehjem ground-glass tissue grinder cooled with crushed ice and centrifuged at $1300 \times g$ for 20 minutes.[6] Aliquots of supernatant fluid were taken for lipase and amylase determinations. To activate trypsinogen and chymotrypsinogen

[5] See footnote 3.

[6] Since the analytical work was completed, it has been reported that addition of 0.1% Triton X-100 to the 0.15 M NaCl gave more complete extraction of trypsinogen and chymotrypsinogen into the supernatant fraction from the pancreatic homogenates (Gorrill, A. D. L., and J. W. Thomas 1967 Anal. Biochem., *19*: 211). But the saline extraction without Triton X-100, which gave comparable extraction of enzymes, was adequate for comparison of different experimental treatments as indicated by Gorrill and Thomas 1967 J. Nutr., *92*: 215.

TABLE 1

Composition of diets

	Water-soluble diet (L-479 E9)	Semipurified diet (L-474 E12)
	g/100 g solids	*g/100 g solids*
Amino acid mixture [1]	22.2	—
Casein	—	24.0
DL-Methionine	—	0.3
Glucose	70.5	—
Starch	—	60.4
Cellophane spangles	—	5.0
i-Inositol	—	0.1
Corn oil	—	3.0
Ladek-55 (fat-soluble vitamins) [2]	—	2.0
Ethyl linoleate and fat-soluble vitamins [3]	—	—
Vitamin B mixture	0.32 [4]	0.5 [5]
Mineral mixture	7.00 [6]	4.7 [7]

[1] Amino acid mixture contained: (in grams) L-lysine·HCl, 1.25; L-histidine·HCl·H_2O, 0.55; L-tryptophan, 0.40; L-phenylalanine, 0.90; L-isoleucine, 0.50; L-leucine, 0.80; L-threonine, 0.50; L-methionine, 0.85; L-valine, 0.70; L-arginine, 0.75; L-asparagine, 1.20; L-proline, 3.00; monosodium L-glutamate, 6.00; glycine, 0.50; L-serine, 1.55; L-alanine, 0.75; and tyrosine-ethylester·HCl, 2.00.

[2] Ladek-55 contained: *dl*-α-tocopheryl acetate, 10 mg; vitamin A conc, natural ester form, 1600 IU; vitamin D_3, 100 IU; vitamin E, mixed tocopherols, 37.5 mg; vitamin K_3, 10 mg; and corn oil to make 2 g.

[3] Ethyl linoleate and fat-soluble vitamins were administered orally to the group fed the water-soluble diet at 200 mg/day per animal. Daily supplement contained: (in milligrams) *dl*-α-tocopheryl acetate, 4.0; vitamin A palmitate, 0.33; vitamin K_1, 0.55; vitamin D_3, 0.00035; and ethyl linoleate to make 200 mg.

[4] B-vitamin mixture contained: (in milligrams) thiamine·HCl, 0.50; riboflavin, 0.75; pyridoxine·HCl, 0.63; niacin, 3.75; inositol, 25; Ca pantothenate, 5.0; *p*-aminobenzoic acid, 30; biotin, 0.10; folic acid, 0.15; cyanocobalamin, 0.03; and choline chloride, 250.

[5] B-vitamin mixture contained: (in milligrams) thiamine, 6; riboflavin, 3; nicotinamide, 5; calcium pantothenate, 30; choline chloride, 200; pyridoxine·HCl, 2; pyridoxamine dihydrochloride, 0.4; biotin, 0.1; folic acid, 1; *p*-aminobenzoic acid, 5; 0.1% trituration B_{12} in mannitol, 25; and rice starch carrier to 500.

[6] Mineral mixture contained: (in milligrams) ferrous gluconate, 35; KI, 3.0; $Mn(C_2H_3O_2)_2 \cdot 4H_2O$, 26; $ZnSO_4 \cdot H_2O$, 5.5; $Cu(C_2H_3O_2)_2 \cdot H_2O$, 2.5; $Co(C_2H_3O_2)_2 \cdot 4H_2O$, 0.9; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.6; Na_2SeO_3, 0.011; $Cr(C_2H_3O_2)_2 \cdot H_2O$, 0.48; calcium fructose 1,6-diphosphate, 5000; magnesium fructose 1,6-diphosphate, 500; NaCl, 380; and CH_3COOK, 1060.

[7] Mineral mixture contained: (in milligrams) NaCl, iodized, 515; $MgSO_4$, 400; Fe $(C_6H_5O_7)_2$, 60; $MnCO_3$, 20; CuO, 2.5; $CoCl_2 \cdot 6H_2O$, 0.05; NaF, 0.01; MoO_3, 0.005; KBr, 0.01; Na_2SeO_3, 0.01; $CaCO_3$, 1700; K_2HPO_4, 1000; and Na_2HPO_4, 1000.

in the pancreas, 2 ml of the above supernatant fluid were mixed with 8 ml of 0.1 M Tris buffer at pH 7.2 containing 0.05 M $CaCl_2$, and incubated with 15 mg purified enterokinase at 4° for 20 hours. The contents of the small intestine, large intestine and cecum were homogenized separately in a mixer [7] cooled with crushed ice.

Activated pancreatic extract, pancreatic solution intended for lipase and amylase determinations and homogenized intestinal contents were centrifuged at 20,000 × *g* for 20 minutes at 4°, and the supernatant fluids were used for enzyme determinations after the appropriate dilutions.

Lipase and amylase activities were measured in serum, pancreas and intestinal contents, and trypsin and chymotrypsin activities were determined in pancreas and intestinal contents. Lipase activity was determined by the methods of Roe and Byler (17) and Roe and Franklin (18) using olive oil emulsified with acacia in a 0.02 M Tris buffer solution (pH 8.5) as a substrate. The fatty acid liberated in the hydrolysis was measured by titration. One enzyme unit is defined as μmoles of fatty acid liberated per minute at 37°. Amylase activity was determined according to the method of Dahlqvist (19) with slight modifications, using starch as a substrate. The incubation of substrate and supernatant fluid (enzyme source) was carried out for 30 minutes at 37° instead of 3 minutes at 25°. The maltose liberated from starch was measured using 3,5-dinitrosalicylate reagent. One unit of amylase is the activity liberating reducing groups corresponding to 1 μmole maltose/minute at 37°.

Trypsin and chymotrypsin activities were assayed by the method of Hummel (20) as outlined by Worthington Biochemical Corporation (21) using *p*-tosyl-arginine methyl ester hydrochloride (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE) as substrates, respectively. The rate of hydrolysis of TAME or BTEE was measured by the increase in absorbancy at 247 mμ (25° and pH 8.1), or at 256 mμ (25° and pH 7.8), respectively, using a spectrophotometer with a recorder.[8] One enzyme unit is equal to 1 μmole of respective substrate hydrolyzed per minute.

The protein content of the supernatant was determined by the method of Lowry et al. (22) using bovine serum albumin as the standard. Enzyme activities in pancreas and intestinal contents were calculated in terms of units per milligram of protein, per unit wet weight, per 100 g body weight and per organ. The relative magnitude, variability and interpretation of the data

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

[8] Beckman DB-G, Beckman Instruments, Inc., Fullerton, Calif.

were similar regardless of the way in which results were expressed. Enzyme activities are reported as units per 100 ml serum, total units per pancreas per 100 g body weight, total units in intestinal tract and units per gram of intestinal content. The data were analyzed statistically by the *t* test to compare dietary treatments within germfree and also within conventional rats, between germfree rats fed two different diets, and also between conventional rats fed these diets.

For histology, pancreatic glands were fixed in formalin. Pancreatic glands were washed and dehydrated in ethyl alcohol, cleared in xylene and embedded in paraffin. Sections were cut at 7 μ and were stained with hematoxylin and eosin for light microscopic examination.

RESULTS

The body weights and pancreas weights of germfree (gf) and conventional (conv) rats fed liquid (L) diet were slightly lower than in respective groups fed semipurified (S) diet (mean body weights ± SEM: gf L, 294 ± 7; conv L, 314 ± 5; gf S, 319 ± 5; conv S, 331 ± 10, and mean pancreas weights ± SEM: gf L, 1.11 ± 0.04; conv L, 1.24 ± 0.05; gf S, 1.25 ± 0.04; conv S, 1.40 ± 0.03). Germfree rats fed either diet showed a slight decrease in body and pancreas weights as compared with conventional rats. There was no difference, however, in pancreas weights between dietary treatment or between germfree and conventional status when values were expressed per 100 g body weight (gf S, 0.38 ± 0.01; conv S, 0.40 ± 0.01; gf L, 0.38 ± 0.014; conv L, 0.39 ± 0.014).

Histopathological examination of sections of pancreas from the germfree and conventional rats fed liquid diet showed that the pancreatic acinar cells in all sections were normal. There were no microscopic lesions indicative of fibrosis, atrophy, degranulation of the acinar cells and of fatty infiltration.

Tables 2 and 3 present the lipase and amylase levels, respectively, in the serum, pancreas and intestinal contents of germfree and conventional rats fed liquid or semipurified diet. Lipase (table 2) and amylase (table 3) levels in serum and pancreas were not affected either by germfree status or by diet. There was a slight, but not significant, decrease in lipase and amylase levels in the small intestinal contents of germfree and conventional rats fed liquid diet as compared with animals fed the semipurified diet. Feeding of liquid diet was generally associated with a lower activity of the enzymes in the contents of the cecum and large intestine. In the large intestine, however, the difference in lipase activity between conventional rats fed liquid diet and those fed semipurified diet did not reach statistical significance. The presence of a microflora was associated with a decrease of amylase levels in the contents of the small intestine and with a considerable decrease in both lipase and

[9] See footnote 3.

TABLE 2

Effect of feeding water-soluble and semipurified diets to germfree and conventional rats on lipase activity [1] in the pancreas and intestinal contents

	Germfree		Conventional	
	Water-soluble	Semipurified	Water-soluble	Semipurified
Serum, units/100 ml	10.5 ± 0.80 [2]	10.4 ± 0.55	11.2 ± 0.81	9.9 ± 0.38
Pancreas, units/100 g body wt	493 ± 35	560 ± 62	599 ± 59	545 ± 51
Small intestine, total units	159 ± 27	187 ± 21	143 ± 18	185 ± 25
units/g contents	85.9 ± 14.6	96.4 ± 10.8	71.5 ± 9	96.4 ± 13
Cecum, total units	55.2 ± 3.7 [3,5]	81.3 ± 7.4 [5]	2.10 ± 0.25 [3]	5.22 ± 0.25
units/g contents	4.68 ± 0.31 [3,5]	7.46 ± 0.68 [5]	0.75 ± 0.09 [3]	2.37 ± 0.11
Large intestine, total units	5.00 ± 1.09 [4,5]	14.9 ± 2.72 [6]	1.11 ± 0.31	1.74 ± 0.56
units/g contents	4.03 ± 0.88 [4,5]	10.6 ± 1.94 [6]	0.74 ± 0.21	1.09 ± 0.35

[1] Unit of activity is expressed as micromoles of fatty acid liberated per minute.
[2] Averages of 7 to 11 animals ± SE of the mean.
[3,4] Difference between dietary groups within germfree or conventional status significant, $P < 0.01$ and $P < 0.05$, respectively.
[5,6] Difference from the conventional group fed similar diet significant, $P < 0.01$, $P < 0.05$, respectively.

TABLE 3

Effect of feeding water-soluble and semipurified diets to germfree and conventional rats on amylase activity [1] in the pancreas and intestinal contents

	Germfree		Conventional	
	Water-soluble	Semipurified	Water-soluble	Semipurified
Serum, units/100 ml	1.38 ± 0.07 [2]	1.37 ± 0.05	1.30 ± 0.06	1.49 ± 0.09
Pancreas, units/100 g body wt	1374 ± 76	1545 ± 134	1556 ± 92	1407 ± 125
Small intestine, total units	616 ± 74 [3]	863 ± 117 [4]	333 ± 51	369 ± 38
units/g contents	333 ± 40 [3]	445 ± 60 [4]	167 ± 25	192 ± 20
Cecum, total units	70.9 ± 7.3 [4,5]	110 ± 13.8 [4]	2.38 ± 0.34 [6]	13.2 ± 1.6
units/g contents	6.01 ± 0.62 [4,5]	10.1 ± 1.27 [4]	0.85 ± 0.12 [6]	6.0 ± 0.73
Large intestine, total units	6.1 ± 1.31 [4,5]	24.7 ± 4.47 [4]	0.95 ± 0.13 [6]	2.64 ± 0.13
units/g contents	4.92 ± 1.06 [4,5]	17.6 ± 3.19 [4]	0.63 ± 0.09 [6]	1.65 ± 0.08

[1] Unit of activity is expressed as micromoles × 10^3 of maltose liberated per minute.
[2] Averages of 7 to 11 animals ± SE of the mean.
[3,4] Difference from the conventional group fed similar diet significant, $P < 0.05$, $P < 0.01$, respectively.
[5,6] Difference between dietary groups within germfree or conventional status significant, $P < 0.05$, $P < 0.01$, respectively.

TABLE 4

Effect of feeding water-soluble and semipurified diets to germfree and conventional rats on trypsin activity [1] in the pancreas and intestinal contents

	Germfree		Conventional	
	Water-soluble	Semipurified	Water-soluble	Semipurified
Pancreas, units/100 g body wt	720 ± 71 [2,3]	943 ± 66	749 ± 63 [3]	955 ± 60
Small intestine, total units	308 ± 41 [4,5]	448 ± 23 [6]	205 ± 16 [3]	267 ± 20
units/g contents	166 ± 22 [4,5]	231 ± 12 [6]	103 ± 8 [3]	139 ± 10
Cecum, total units	298 ± 22 [4,6]	424 ± 19 [6]	18.1 ± 1.67 [4]	31.2 ± 3.07
units/g contents	25.3 ± 1.86 [4,6]	38.9 ± 1.74 [6]	6.46 ± 0.60 [4]	14.2 ± 1.39
Large intestine, total units	23.9 ± 2.44 [4,6]	45.2 ± 4.54 [6]	8.50 ± 0.68 [4]	14.8 ± 1.10
units/g contents	19.3 ± 1.97 [4,6]	32.3 ± 3.24 [6]	5.67 ± 0.45 [4]	9.25 ± 0.69

[1] Unit of activity is expressed as micromoles of TAME hydrolyzed per minute.
[2] Averages of 7 to 11 animals ± SE of the mean.
[3,4] Difference between dietary groups within germfree or conventional status significant, $P < 0.05$, $P < 0.01$, respectively.
[5,6] Difference from the conventional group fed similar diet significant, $P < 0.05$, $P < 0.01$, respectively.

TABLE 5

Effect of feeding water-soluble and semipurified diets to germfree and conventional rats on chymotrypsin activity [1] in pancreas and intestinal contents

	Germfree		Conventional	
	Water-soluble	Semipurified	Water-soluble	Semipurified
Pancreas, units/100 g body wt	896 ± 65 [2,3]	1194 ± 104	913 ± 42 [4]	1072 ± 28
Small intestine, total units	480 ± 31 [4,5]	594 ± 17 [5]	246 ± 16 [4]	335 ± 20
units/g contents	259 ± 17 [4,5]	306 ± 8.8 [4]	123 ± 8 [4]	174 ± 10
Cecum, total units	133 ± 9.7 [4,5]	189 ± 12.4 [5]	8.53 ± 0.92 [4]	14.3 ± 1.29
units/g contents	11.3 ± 0.82 [4,5]	17.3 ± 1.14 [5]	3.04 ± 0.33 [4]	6.5 ± 0.59
Large intestine, total units	11.8 ± 0.74 [4,5]	20.1 ± 1.42 [5]	5.16 ± 0.36 [4]	7.93 ± 0.48
units/g contents	9.5 ± 0.60 [4,5]	14.4 ± 1.01 [5]	3.44 ± 0.24 [4]	4.96 ± 0.30

[1] Unit of activity is expressed as micromoles of BTEE hydrolyzed per minute.
[2] Averages of 7 to 11 animals ± SE of the mean.
[3,4] Difference between dietary groups within germfree or conventional status significant, $P < 0.05$, $P < 0.01$, respectively.
[5] Difference from the conventional group fed similar diet significant, $P < 0.01$.

amylase levels in cecum and large intestine.

The data summarized in tables 4 and 5 indicated that germfree status had no effect on the activities of trypsinogen (table 4) and chymotrypsinogen (table 5) within the pancreas, whereas feeding of liquid diet resulted in lower activities of these enzymes in both germfree and conventional rats compared with animals fed a semi-

purified diet. Both germfree and conventional rats fed liquid diet demonstrated lower activities of these proteolytic enzymes in the contents of the small intestine, cecum and large intestine. The presence of microflora in the intestinal tract was associated with considerably lower enzyme activities in the contents of the small intestine, cecum and large intestine.

In general, the results indicate that in the absence of natural exogenous substrates in the diet the rate of inactivation of lipase, amylase, trypsin and chymotrypsin in the contents as they pass from small intestine to cecum or large intestine, or both, is increased. The data also demonstrated that most nonbacterial and bacterial inactivation of the pancreatic enzymes occurred in the cecum. Comparison of small intestinal and cecal concentrations of these enzymes indicated trypsin as the most stable enzyme and amylase as the least stable of the group.

DISCUSSION

The pancreatic changes produced in rats by feeding diets deficient in specific essential amino acids or in protein (total nitrogen) are characterized by atrophy and degranulation of acinar cells and sometimes by fatty infiltrations and diffuse fibrosis (5). These conditions which result in disruption of normal pancreatic structure are reflected in changes in the levels of trypsinogen, chymotrypsinogen, lipase and amylase in the pancreas, and of lipase and amylase in serum (6, 23). The present study revealed neither microscopic nor macroscopic structural changes in the pancreas, nor changes in the levels of serum lipase and amylase in either germfree or conventional rats fed the liquid diet. These results indicate that no impairment of functional capacity of the pancreas occurred as a result of feeding the liquid diet. They provide additional evidence that the liquid diet used in this investigation was nutritionally adequate for germfree and conventional rats.

Our histopathological observations did not confirm the preliminary observations of Geever et al.[10] who reported that a chemically defined, liquid diet produced atrophic and fibrotic changes in the pancreas of germfree rats. These investigators, however, fed a liquid diet lacking in chromium and selenium and containing an emulsifying agent, Tween 80. Mertz (24) has shown the importance of chromium as a trace element to enhance insulin activity and prevent overt disturbances of carbohydrate metabolism, and concomitant chronic diseases resulting from a low chromium state. Insulin appeared to be specifically involved, by a still unknown mechanism, in the biosynthesis of pancreatic amylase (25). In addition, emulsifying agents such as Tween 80 are known to affect certain enzyme activities in vitro and in vivo (26, 27). The atrophic and fibrotic changes observed by Geever et al.[11] in germfree rats fed liquid diet may have been related to any one of the above factors.

The observations of Ben Abdeljlil and Desnuelle (12) indirectly indicate that the exocrine pancreas received its information to synthesize digestive enzymes from the products formed during the digestion process, rather than from the ingested products as such. In the present study, the production of comparable levels of lipase, amylase, trypsinogen and chymotrypsinogen in the pancreas of germfree and conventional rats fed a completely defined liquid diet (amino acids, glucose and ethyl linoleate) free from dietary substrates (intact proteins or peptides, starch and triglyceride), compared with those animals fed a semipurified diet containing dietary substrates, provided direct evidence for the above hypothesis.

The difference in response in the pancreatic levels of trypsinogen and chymotrypsinogen on the one hand, and amylase and lipase on the other hand to different diets indicates that the mechanisms regulating the adaptive (dietary) response of various pancreatic enzymes are not identical. Our observations are consistent with those of Howard and Yudkin (11) who found a decrease in trypsinogen activity and no change in amylase levels in rats fed a hydrolyzed casein diet as compared with an intact casein diet. A decrease in the activities of trypsinogen and chymotrypsinogen observed in rats fed the liquid diet might mean that the amino

[10] See footnote 3.
[11] See footnote 3.

acids or other materials reaching the pancreas for these enzyme syntheses when the intact protein (casein) was fed might not be in the optimal proportion as those reaching it when an amino acid diet was fed. This, in turn, might result in a difference in amino acid pools in the pancreas of rats fed the liquid diet or the semipurified diet.

In agreement with the findings of Borgstrom et al. (28), Pelot and Grossman (29) and Snook (9), the pancreatic enzymes were found to undergo a progressive inactivation on entering the small intestine and subsequently passing through the entire intestinal tract. Comparison of enzyme activities in the contents of the small intestine, cecum and large intestine of rats fed the liquid and semipurified diets provided information about the dietary influence on this inactivation. In the case of germfree rats, where bacterial inactivation is excluded, the data indicate generally less destruction of enzymes as they pass from small intestine to cecum and large intestine in animals fed the semipurified diet than in rats fed the liquid diet. The data obtained with conventional rats further confirmed this conclusion. The mechanisms involved in this phenomenon are, however, speculative. The protective effect of the semipurified solid diet on trypsin and chymotrypsin activities may be explained by the intact dietary protein retarding the rate of breakdown of these enzymes by competing with the enzymes as substrates for proteolysis. In the case of lipase and amylase, we should not rule out the possibility of formation of enzyme-substrate complexes (binding of enzyme to substrate) which might protect lipase and amylase from proteolysis. It is also possible that the concentration of these enzymes (lipase, amylase, trypsin and chymotrypsin) in intestinal contents could be influenced by the rate of passage of chyme through the intestinal tract. This rate of passage could, in turn, be influenced by the type of diet fed or the microbial status of the animal. An elucidation of the factors involved in the inactivation of these enzymes under in vivo conditions will depend on further work.

A comparison of lipase, amylase, trypsinogen and chymotrypsinogen levels in the pancreas, and also lipase and amylase levels in the serum of germfree and conventional rats indicated that the absence of microbial effects in the system did not appreciably change the overall production of these enzymes by the pancreas, or their level of circulation in the serum. The markedly lower level of all pancreatic enzymes in the intestinal contents of conventional rats compared with those of germfree rats provided a definite indication of a microbial factor in the inactivation of these enzymes. This observation was further strengthened by the observation that, in the contents of the cecum and large intestine where there is extensive microbial activity, the difference in pancreatic enzyme content between germfree and conventional rats was especially marked. Recently, Lepkovsky et al. (30) reported that there was little difference between germfree and conventional rats in the concentration of lipase, amylase and proteases in the pancreas and in intestinal contents. These observations are at variance with our observations and might be due to shipment of samples before analysis, and to use of different assay techniques. Trypsin activity was measured with less specific substrates such as casein and hemoglobin, and could represent a sum of the activities of bacterial and intestinal proteases (9). Despite this, the data presented by Lepkovsky et al. (30) appear to indicate that germfree animals have somewhat higher levels of protease activity in the contents of the lower third of the small intestine, the cecum and the colon than do their conventional counterparts.

In conclusion, this investigation indicated that the liquid diet used in the study was nutritionally adequate for germfree and conventional rats especially as far as normalcy and function of the pancreas were concerned. Diets with and without dietary substrates maintain the production of digestive enzymes in the pancreas. Both diet and intestinal microflora, however, play an important role in subsequent inactivation of these enzymes in the intestinal tract.

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Role of Selenium in the Nutrition of the Chick [1,2]

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ABSTRACT In an attempt to establish that selenium is an essential trace element for chicks and not merely a substitute for vitamin E, the effects of feeding various diets low in selenium have been investigated. Chicks were protected against exudative diathesis by supplements to semipurified diets of either 10 ppm *d*-α-tocopheryl acetate or 0.04 ppm selenium. In contrast, chicks given diets prepared with crystalline amino acids and containing less than 0.005 ppm selenium had poor growth and high mortality even when the diet contained up to 200 ppm *d*-α-tocopheryl acetate. Higher levels of vitamin E prevented mortality but even with 1000 ppm growth was inferior to that obtained with supplements of selenium and no added vitamin E. A relationship was demonstrated between the selenium requirement and the level of vitamin E in the diet. Using diets containing 100 ppm vitamin E, the selenium requirement was less than 0.01 ppm, whereas with 10 ppm vitamin E it was more than 0.02 ppm and with no added vitamin E, approximately 0.05 ppm.

Diets containing low levels of both vitamin E and selenium have been shown to produce nutritional disorders in several species, including rats (1, 2), chickens (3, 4) and turkeys (5–7). Similar diseases have been found to occur in farm livestock receiving feeds low in selenium (8–10).

These disorders can be prevented either by adding a small amount of selenium to the diet or by providing vitamin E. There is scant evidence for the existence of selenium-responsive disease in animals receiving a normal allowance of vitamin E, and thus the status of selenium as an essential nutrient has remained tenuous (11).

Chicks fed diets containing low levels of selenium and vitamin E develop a fatal disease, exudative diathesis, in which there are massive accumulations of fluid in the muscles and connective tissues. This condition can be prevented by supplementing the diet with either selenium (3, 4, 12) or 5 to 20 ppm *d*-α-tocopheryl acetate [3,4] (13). As selenium is present in almost all plant and animal proteins, diets low in selenium used in nutritional experiments are unlikely to be completely free of this element. It is therefore conceivable that animals have a nutritional need for selenium which, in the presence of vitamin E, is so small that in most experiments it has been met by selenium contaminating the basal diets. Evidence to support such a possibility was obtained by Nesheim and Scott (12), using a diet which was considered to be especially low in selenium. They found that chicks needed selenium for growth and survival even when the diet was supplemented with 80 ppm *d*-α-tocopheryl acetate. Unfortunately, the effect could not be reproduced in other experiments with new ingredients, and thus could not be investigated further.

The present paper describes an investigation of the nutritional role of selenium in which chicks were rigorously deprived of selenium, using diets made up of crystalline amino acids. The results confirm that selenium is an essential nutrient for the chick, and it is concluded that the nutritional significance of selenium is more than that of a mere substitute for vitamin E.

EXPERIMENTS AND RESULTS

Selenium-depleted chicks. Plymouth Rock × Vantress chicks were obtained from hens fed a diet low in selenium. The diet contained: (in percent) glucose,[5] 50;

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[5] Cerelose, Corn Products Company, Argo, Ill. Cornstarch was sometimes used instead of glucose.

soybean meal,[6] 40; soybean oil, 3; DL-methionine, 0.5; vitamin mixture,[7] 0.5; and salts N (14), 6. At the onset of egg production, $CaCO_3$ (4.5%) and $CaHPO_4 \cdot 2H_2O$ (0.5%) were included at the expense of glucose.

Experimental diets. Diet YCS (table 1) was similar to that of Hathcock et al. (15) except that sodium selenite was omitted and the methionine supplement was increased to 0.6%. Diet YCS-CO was identical to diet YCS with stripped corn oil substituted for stripped lard. Diet A (table 2) was based on the amino acid reference diet of Dean and Scott (16). Cystine was replaced by extra DL-methionine in an attempt to keep contamination with selenium as low as possible. Selenium supplements were added to the diets as sodium selenite.

Selenium content of basal diets. Selenium levels were measured in the basal diets by the method of Allaway and Cary (17). Samples of diet YCS contained 0.02 to 0.035 ppm selenium. Most of the amino acids used in experiments 3, 4, 5 and 7 were from one source,[8] and selenium was not detected (i.e., < 0.005 ppm) in diet A prepared with these ingredients. Amino acids from a different source [9] were used in experiments 6 and 8 and the basal diets were found to contain 0.01 to 0.02 ppm selenium. Analysis of the amino acids revealed comparatively high levels of selenium in L-leucine (0.16 ppm), L-tyrosine (0.16 ppm) and L-phenylalanine (0.06 ppm). A large proportion of the contamination was inexplicable.

TABLE 1

Composition of diet YCS

Ingredients	%
Casein [1]	6.00
Isolated soybean protein [2]	7.00
Torula yeast [3]	10.00
Glucose monohydrate [4]	57.10
Cellulose [5]	3.00
Stripped lard [6]	5.00
Vitamin mixture [7]	0.89
Mineral mixture [8]	6.04
Amino acid mixture [9]	4.97

[1] Sheffield Chemical Company, Norwich, N. Y.
[2] Ralston Purina Company, St. Louis, Mo.
[3] Lake States Yeast, Rhinelander, Wis.
[4] Cerelose, Corn Products Company, Argo, Ill.
[5] Solka-Floc, Brown Company, Berlin, N. H.
[6] From Distillation Products Industries, Rochester, N. Y.
[7] The vitamin mixture supplied the following per kilogram of diet: (in milligrams) thiamine, 10; riboflavin, 10; pyridoxine·HCl, 4.5; nicotinic acid, 50; folic acid, 4; Ca pantothenate, 20; menadione, 1; choline chloride, 1490; and ethoxyquin, 125; (in micrograms) biotin, 200; and vitamin B_{12}, 20; (in International Units) vitamin A, 5600; and vitamin D_3, 3110.
[8] The mineral mixture supplied the following per kilogram of diet: (in grams) $CaHPO_4 \cdot 2H_2O$, 27.22; $CaCO_3$, 13.55; KH_2PO_4, 8.68; NaCl, 7.13; and $KHCO_3$, 2.10; (in milligrams) $Cu(acetate)_2 \cdot H_2O$, 34; $MnCl_2 \cdot 4H_2O$, 466; $FePO_4 \cdot 4H_2O$, 267; MgO, 850; KI, 2.6; ZnO, 69; $CoCl_2 \cdot 6H_2O$, 1.7; $Na_2MoO_4 \cdot 2H_2O$, 8.3; and $Cr_2K_2(SO_4)_4 \cdot 24H_2O$, 48.
[9] The amino acid mixture supplied the following per kilogram of diet: (in grams) L-arginine·HCl, 10; glycine, 14; L-glutamic acid, 16; L-lysine·HCl, 2.5; L-tyrosine, 1.5; L-tryptophan, 1.2; L-phenylalanine, 0.5; L-leucine, 1.5; L-isoleucine, 0.5; and DL-methionine, 6.0.

TABLE 2

Composition of basal diet A

Ingredients	Amount
	%
Amino acid mixture [1]	23.53
Sucrose	60.07
Soybean oil	5.00
Vitamin mixture [2]	0.50
Mineral mixture [3]	7.70
Cellulose	3.00
Choline chloride	0.20
	ppm
Antioxidant [4]	125

[1] Contained: (in grams) L-arginine·HCl, 1.33; L-glutamic acid, 12; glycine, 1.6; L-histidine, 0.3; L-isoleucine, 0.8; L-leucine, 1.2; L-lysine·HCl, 1.4; DL-methionine, 0.9; L-phenylalanine, 0.68; L-proline, 1; L-threonine, 0.65; L-tryptophan, 0.22; L-tyrosine, 0.63; and L-valine, 0.82.
[2] A mixture in glucose containing: (in milligrams) inositol, 25; nicotinic acid, 5; calcium pantothenate, 3; thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; vitamin A palmitate, 1; folic acid, 0.4; menadione, 0.12; biotin, 0.011; cholecalciferol, 0.002; and vitamin B_{12}, 0.0014.
[3] $CaCO_3$, 1.5; $CaHPO_4 \cdot 2H_2O$, 3.4; $NaHCO_3$, 1.1; $KHCO_3$, 1.1; $MgCO_3$, 0.5; $FePO_4 \cdot 2H_2O$, 0.03; $MnSO_4 \cdot H_2O$, 0.05; KIO_3, 0.001; $ZnCO_3$, 0.015; $CuCl_2 \cdot 2H_2O$, 0.003; $Na_2MoO_4 \cdot 2H_2O$, 0.0008; $CoCl_2 \cdot 6H_2O$, 0.0002; and $Cr_2K_2(SO_4)_4 \cdot 24H_2O$, 0.001.
[4] Santoquin, Monsanto Company, St. Louis, Mo.

Determinations of the Requirements for Selenium and Vitamin E using Semipurified Diets

Diet YCS is a semipurified diet low in selenium and low in vitamin E. It was used in experiments 1 and 2 to verify, with selenium-depleted chicks, previous findings concerning the requirement for selenium and its relationship to the need for vitamin E. In these experiments chicks were

[6] Most of the selenium in this diet was contributed by the soybean meal, which contained approximately 0.1 ppm.
[7] As described in footnote 2, table 2, with 10 mg *dl*-α-tocopheryl acetate.
[8] Mann Assayed, Mann Research Laboratories, Inc., New York.
[9] Nutritional Biochemicals Corporation, Cleveland, Ohio.

fed the diets supplemented as indicated below, from hatching, and mortality and signs of disease were recorded. At 24 days of age surviving chicks were killed and examined for signs of exudative diathesis.

Experiment 1. The requirement for selenium was determined in chicks fed basal diet YCS unsupplemented with vitamin E. Groups containing 20 chicks were given unsupplemented diet (group 1), and diet supplemented with selenium at the levels (in ppm) 0.01 (group 2), 0.02 (group 3), 0.03 (group 4) and 0.04 (group 5).

The results indicated that it was necessary to supplement the basal diet with at least 0.04 ppm selenium as selenite to prevent exudative diathesis and mortality (table 3).

Experiment 2. The requirement for vitamin E was determined in chicks fed diet YCS unsupplemented with selenium. The experiment was repeated with basal diet YCS-CO in which stripped corn oil was used in place of stripped lard. This substitution, in substantially increasing the level of unsaturated fatty acids, resulted in a basal semipurified diet which had a fatty acid content similar to that of the crystalline amino acid diets used in other experiments. Groups of 15 or 16 chicks were fed the basal YCS diet unsupplemented (group 6) or supplemented with *d*-α-tocopheryl acetate at the levels (in ppm) 2 (group 7), 5 (group 8), 10 (group 9) and 50 (group 10). Other groups of chicks were given the basal diet YCS-CO unsupplemented (group 11) or supplemented with *d*-α-tocopheryl acetate at the levels (in ppm) 5 (group 12), 10 (group 13), 50 (group 14), 100 (group 15) and 200 (group 16). Group 17 was given basal diet YCS-CO supplemented with 0.1 ppm selenium.

TABLE 3

Requirement for selenium for protection against exudative diathesis in chicks fed basal diet YCS (exp. 1)

Group	Selenium supplement [1]	No. of chicks used	No. of chicks at day 24	
			Alive	Normal [2]
	ppm Se			
1	—	20	2	0
2	0.01	20	9	0
3	0.02	20	14	6
4	0.03	20	15	13
5	0.04	20	20	20

[1] Added as sodium selenite.
[2] No signs of exudative diathesis.

With no added selenium, protection against exudative diathesis was obtained on addition of 10 ppm of vitamin E to basal diet YCS (table 4). The use of a basal diet containing stripped corn oil instead of stripped lard (diet YCS-CO) increased the severity of the disease in the chicks receiving inadequate levels of vitamin E, but it did not substantially increase the minimum level of vitamin E necessary for protection.

Thus, a requirement for selenium could be demonstrated in chicks given semipurified diets which lacked vitamin E, but, as found by previous investigators, this need for selenium disappeared when the diet was fortified with 10 to 50 ppm vitamin E.

The Selenium-responsive Diseases in Chicks Fed Amino Acid Diets

In an attempt to obtain a chick diet which contained very small quantities of selenium, diet A was prepared using crystalline amino acids as the sole source of protein nitrogen. When tested with selenium-depleted chicks, the diet did not appear to support satisfactory growth even when fortified with high levels of vitamin E, unless selenium supplements were pro-

TABLE 4

Requirement for vitamin E for protection against exudative diathesis in chicks fed basal diets YCS and YCS-CO (exp. 2)

Group	Vitamin E supplement [1]	No. of chicks used	No. of chicks at day 24	
			Alive	Normal [2]
	ppm			
	Stripped lard basal diet (YCS)			
6	0	16	2	0
7	2	16	7	3
8	5	16	15	14
9	10	16	16	14
10	50	16	16	16
	Stripped corn oil basal diet (YCS-CO)			
11	0	16	0	0
12	5	16	8	4
13	10	16	13	10
14	50	15	14	14
15	100	16	16	16
16	200	15	15	15
17	0 [3]	15	15	15

[1] Added as *d*-α-tocopheryl acetate.
[2] No signs of exudative diathesis.
[3] Given supplement of 0.1 ppm selenium as sodium selenite.

vided. The amount of selenium required depended on the vitamin E content of the diet. These effects are demonstrated in experiments 3, 4 and 5 in which chicks were reared on basal diet A supplemented as indicated below. The chicks were weighed at intervals, and mortality and signs of disease were noted.

Experiment 3. Groups of 12 chicks were fed basal diet A fortified with 100 ppm *d*-α-tocopheryl acetate, otherwise unsupplemented or containing 0.1 ppm selenium.

Chicks given the basal diet containing 100 ppm vitamin E (fig. 1) showed poor growth and poor feathering; by day 40 most of them had died. In contrast, the chicks given selenium grew well and were of normal appearance throughout the experiment. Experiment 3 thus confirms the earlier experiments (12) which indicated that with some low selenium diets vitamin E, even at high levels, is unable to maintain growth and survival.

Experiment 4. Groups of 5 chicks were fed basal diet A fortified with 100 ppm *d*-α-tocopheryl acetate, unsupplemented, and supplemented with selenium at the levels (in ppm) 0.01, 0.02, 0.05 and 0.1. The supplements were started when the chicks were 10 days of age.

In this attempt to measure the minimum requirement all selenium-supplemented diets produced equal, satisfactory growth (fig. 2). It is concluded that the selenium requirement of chicks given a diet containing 100 ppm vitamin E is met by 0.01 ppm, or less, of dietary selenium as selenite.

Experiment 5. The requirement for selenium was observed to be higher when the crystalline amino acid diets contained lower amounts of vitamin E than those used in experiments 3 and 4. This was demonstrated by feeding groups of 5 chicks basal diet A fortified with 10 ppm *d*-α-tocopheryl acetate unsupplemented and supplemented with selenium at the levels (in ppm) 0.005, 0.01, 0.02, 0.05 and 0.1.

The results demonstrated (fig. 3) that it was necessary to add 0.02 ppm selenium to maintain growth. This level, however, did not prevent all deficiency signs: one chick in the group given the diet containing 0.02 ppm selenium died and two survivors developed exudative diathesis. The requirement for selenium with 10 ppm vitamin E in the diet was thus above 0.02 ppm but below 0.05 ppm.

Biological Activity of Selenium in the Absence of Vitamin E

Experiment 6. The soybean oil in basal diet A was replaced by stripped corn oil to

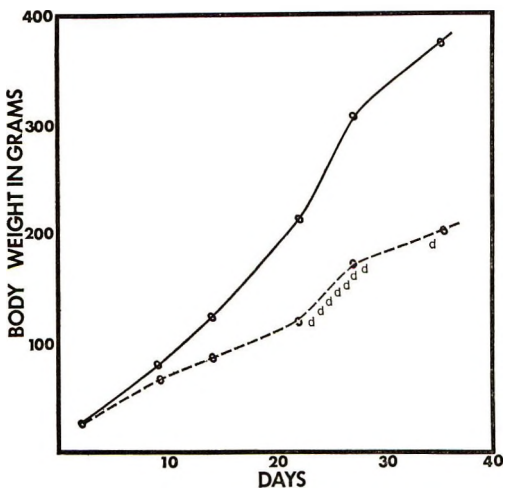

Fig. 1 Mean body weights of groups of 12 chicks (exp. 3) given diet A containing 100 ppm *d*-α-tocopheryl acetate unsupplemented (broken line), or supplemented with 0.1 ppm selenium. Deaths of individual chicks denoted by d.

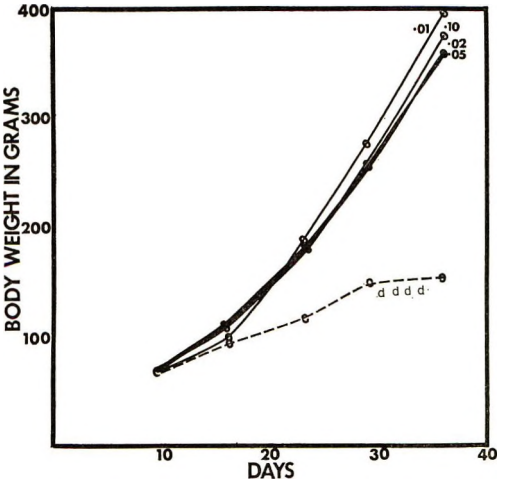

Fig. 2 Mean body weights of groups of five chicks (exp. 4) given diet A containing 100 ppm *d*-α-tocopheryl acetate unsupplemented (broken line), or supplemented with: (in ppm) 0.01, 0.02, 0.05 and 0.1 selenium. Deaths of individual chicks denoted by d.

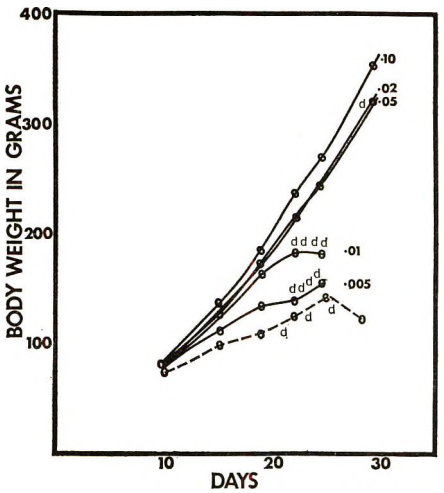

Fig. 3 Mean body weights of groups of five chicks (exp. 5) given diet A containing 10 ppm *d*-α-tocopheryl acetate unsupplemented (broken line), or supplemented with: (in ppm) 0.005, 0.01, 0.02, 0.05 and 0.1 selenium. Deaths of individual chicks denoted by d.

produce a diet with a very low level of vitamin E. In an attempt to destroy any residual tocopherols in the stripped corn oil, the oil used in experiment 6 was heated slowly to 115° with 0.3% lauryl peroxide [10] as recommended by Machlin (8). When the oil had cooled to room temperature, the dietary antioxidant was added and this preparation was mixed with the remaining ingredients of the feed.

Groups of 10 chicks were fed the modified basal diet unsupplemented, and supplemented with selenium at the levels (in ppm) 0.05, 0.1 and 0.5. Other groups were given supplements of (in ppm) 0.05 selenium with 0.5 *d*-α-tocopheryl acetate, and 0.1 selenium with 10 *d*-α-tocopheryl acetate.

Chicks fed the unsupplemented basal diet grew poorly, and all died before 4 weeks of age. All remaining groups, however, had normal growth and appearance. (Details of these results, therefore, are not presented.) Thus, selenium at levels of 0.05 ppm and higher prevented signs of disease even in the absence of vitamin E, and the addition of 0.5 ppm vitamin E to the diet of one group of chicks receiving 0.05 ppm selenium gave no noticeable improvement. Therefore, although the need for selenium increased when the vitamin E intake was reduced, the requirement still did not exceed 0.05 ppm even when vitamin E was completely eliminated from the diet.

Effect of High Levels of Vitamin E on the Need for Selenium

In experiments 7 and 8 the effects of high levels of vitamin E were tested on chicks given basal diet A containing stripped corn oil in place of soybean oil. Experiments 7 and 8 were similar in design but they were conducted at different times using different batches of dietary ingredients. Selenium could not be detected in the basal diet used in experiment 7, whereas the basal diet used in experiment 8 contained 0.018 ppm (see above).

Experiment 7. Groups of 4 chicks were given the basal diet unsupplemented and supplemented with *d*-α-tocopheryl acetate at the levels (in ppm) 5, 10, 20, 50, 100, 200, 500 and 1000. A control group was given the basal diet supplemented with 0.1 ppm selenium.

Experiment 8. Groups of 10 chicks were given the basal diet unsupplemented and supplemented with *d*-α-tocopheryl acetate at the levels (in ppm) 50, 100, 200 and 500. A control group was given the basal diet supplemented with 0.1 ppm selenium.

It was found in experiment 7 (fig. 4) that when the dietary level of vitamin E was 100 ppm or less, growth was poor and the chicks died before 3 weeks of age. By feeding the diet containing 200 ppm vitamin E, mortality was prevented but growth was still poor. At higher levels of vitamin E growth was improved, but was still inferior to that of chicks given the diet supplemented with 0.1 ppm selenium and no vitamin E. In experiment 8, high levels of vitamin E again were found to protect against the deleterious consequences of a lack of selenium (fig. 5), but in this experiment the effect was apparent with lower levels of vitamin E than were found necessary in experiment 7. The difference between the results of the two experiments was probably due to greater contamination with selenium of the basal diet used in experiment 8.

[10] Alperox F, Wallace and Tiernan, Inc., Buffalo, N. Y.

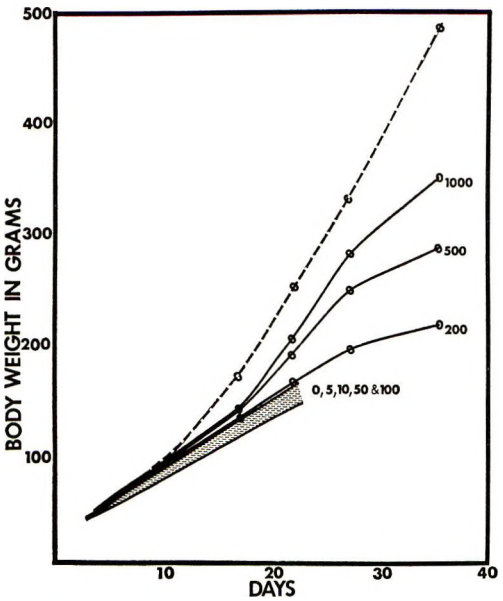

Fig. 4 Mean body weights of groups of four chicks (exp. 7) given diet A containing stripped corn oil and supplements of: (in ppm) zero, 10, 50, 100, 250, 500 and 1000 *d*-α-tocopheryl acetate (solid lines and shaded area), or 0.1 ppm selenium (broken line). Selenium was not detected in the basal diet.

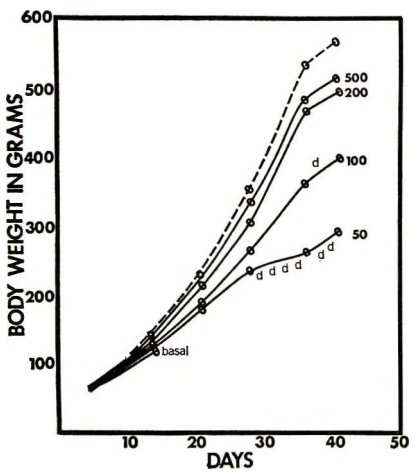

Fig. 5 Mean body weights of groups of 10 chicks (exp. 8) given diet A containing stripped corn oil and supplements of: (in ppm) zero, 50, 100, 200 and 500 *d*-α-tocopheryl acetate (solid line), or 0.1 ppm selenium (broken line). Deaths of individual chicks, except those given the unsupplemented diet which died before 2 weeks of age are denoted by d. The basal diet contained 0.018 ppm selenium.

DISCUSSION

The results of experiments 1 and 2, in demonstrating that supplementation of semipurified diets with vitamin E at 10 ppm eliminates the need for selenium supplements, are in agreement with several similar investigations with semipurified diets conducted in this laboratory during the last decade. This ability of moderate levels of vitamin E to abolish the need for selenium supplements means that the results of experiment 1, and similar investigations, do not provide conclusive evidence of an essential role for selenium. An alternative and equally acceptable interpretation of these experiments with chicks, and many similar investigations involving various species, would be that selenium is merely an unusual substitute for vitamin E.

The main aim of the present series of experiments with highly purified diets was to verify the existence of a selenium requirement in the normal chick. The results definitely establish selenium as a nutritionally essential element. The large intake of vitamin E (100 to 1000 ppm) necessary for the prevention of clinical signs of disease in selenium-deficient chicks considerably exceeds the normal nutritional requirement. Thus, the nutritional role of selenium is more than that of a substitute for a normal intake of vitamin E; on the contrary, it appears more plausible that vitamin E merely reduces the requirement for selenium.

On the basis of these investigations of the selenium requirement, one may calculate that most feeds contain sufficient selenium for them to promote normal growth and prevent exudative diathesis, even if they are unsupplemented with vitamin E; thus, in these roles at least, it is the status of vitamin E as an essential nutrient, and not that of selenium, which is open to question.

The ability of most semipurified low selenium diets such as diet YCS to maintain growth and health when supplemented with vitamin E can be attributed to contamination with selenium at levels above 0.02 ppm. Confirmatory evidence for this explanation was obtained in experiment 4 in which it was found that less than 0.01

ppm selenium as selenite was required in diets containing high levels of vitamin E.

In experiment 8, using a basal diet having a small but detectable contamination with selenium, large amounts of vitamin E were found to be completely effective substitutes for selenium during the period of the assay. Comparison with the results of experiment 7, however, reveals that the trace of selenium in the basal diet used in experiment 8 probably had enhanced considerably the beneficial effects of high levels of vitamin E. The extent to which high levels of vitamin E can substitute for selenium is, as yet, not completely defined, and it is possible that vitamin E would be ineffective at all levels if selenium could be completely eliminated from the basal diets.

Experiments 4 to 8 demonstrate that the requirement for selenium in the chick can vary over a wide range, and that it increases as the dietary level of vitamin E is reduced. This relationship could explain why previous investigations of selenium requirements have been successful when done with diets too low in vitamin E. On the other hand, exudative diathesis and other selenium-responsive disorders in animals severely deficient in vitamin E (1, 2, 5–7) may involve at the fundamental level something other than a simple selenium deficiency. It was noted in the present studies that the abnormalities produced by diets low in vitamin E and moderately low in selenium are unlike those produced by diets containing substantial amounts of vitamin E and extremely small amounts of selenium. In the former, as in experiments 1 and 2, growth is usually good and the most prominent abnormality is exudative diathesis. In the latter (exp. 3), however, the major effect is on growth and feathering, and it is only during the terminal stages of the disease, if at all, that signs of exudative diathesis make their appearance.

The studies reported here did not provide evidence to support any hypothesis that might explain the effect of vitamin E on the selenium requirement of the chick. A simple explanation, compatible with many of the experimental findings, would be that vitamin E somehow affects the utilization of dietary selenium or the formation or stability of an active form. It is also conceivable that vitamin E might alter the metabolism of the chick in such a manner that the demands on some biochemical process involving selenium are changed. These and other theories (19, 20) are plausible, but they lack direct experimental support.

The selenium-vitamin E interrelationship may also involve an effect of selenium on the utilization of vitamin E. Selenium has previously been reported to have an effect on the retention of vitamin E in chicks (21) and rats,[11] and it has been suggested that it may function in a transport system for tocopherols and related lipids (22). Furthermore, selenium-deficient chicks given crystalline amino acid basal diets similar to those employed in the present studies have lower plasma tocopherol levels than controls given selenium.[12] As selenium is biologically active in the absence of vitamin E (exp. 6), however, it is inconceivable that its mode of action could concern only the utilization of vitamin E.

Although it has been established that the chick has a requirement for selenium, the results of these studies show that a quantitative statement of the need cannot be attempted until the requirement for vitamin E is set at a specific level. If vitamin E is omitted from poultry diets, or if it is replaced by a synthetic antioxidant, then the need for selenium is at least 0.05 ppm of diet, and it increases to above 0.1 ppm if there is inefficient absorption. On the other hand, a generous level of vitamin E in the diet would decrease the selenium requirement to 0.02 ppm or even lower. The requirement for selenium thus depends on the definition of a normal dietary level for vitamin E.

The experiments with chicks were undertaken after preliminary tests with quail (23), which also were observed to have a need for selenium even when provided with vitamin E. The demonstration of a need for selenium in chicks and quail receiving vitamin E should encourage similar investigations with other species.

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[12] Unpublished data.

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Changes in Body Weight and Composition of Adult Nongravid Female Rats Deprived of Dietary Protein

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ABSTRACT Seventy-six young adult nongravid female rats of the Carworth strain were used in two experiments to study the effect of dietary protein deprivation on body weight, feed consumption, body composition and serum protein concentration. Semi-purified diets containing glucose, corn oil, vitamins and minerals with or without casein (protein-free = PF, 14% casein = C) were fed ad libitum in both experiments to rats caged in individual wire-bottom cages. Feeding the PF diet for up to 26 days resulted in a continuous weight loss over the entire period. The average loss in body weight as a percentage of initial weight of PF rats in experiments 1 and 2 was 32.9 and 25.7, respectively. Weight loss during 9 days in rats fed the PF diet was restored within 8 days following transfer to the C diet. Feed consumption was reduced only moderately by the PF diet, suggesting a large loss of ingested calories by excretion or metabolism to carbon dioxide and water. Body composition data supported this and indicated a greater relative loss of body energy than of body protein in PF rats. Blood serum protein concentration was reduced moderately ($P < 0.05$) in rats fed the PF diet. The absolute loss of serum protein may have been greater than suggested by the concentration if it is assumed that there was a reduction in blood volume associated with the observed weight loss. Blood serum protein concentration therefore does not appear to be as sensitive an index of protein nutrition in the adult as in the young animal. Final body energy, protein and water content were less in PF rats than in C rats in experiment 2 by an average of 176.5 kcal (36%), 10.9 g (23%) and 41.7 g (28%), respectively. Energy concentration (kilocalories per gram dry weight) was 11% greater in C than in PF rats (5.8 versus 5.2 kcal/g). These data indicate that the adult female nongravid rat can survive a loss of at least one-third of body energy and one-fourth of body protein induced by protein deprivation without developing severe hypoproteinemia, emaciation and edema.

The effect of dietary protein deprivation on reproductive performance has been studied extensively in the rat (1–3) but only limited data are available on its effects on the nongravid female rat. Protein deprivation reduces the incorporation of amino acids into protein in muscle and increases incorporation in liver (4). It results in a three- to fourfold increase in liveramino acid-activating enzymes (5). The nitrogen concentration of liver from protein-deprived rats is reduced and body weight loss is greater than in rats fed an equal intake of an adequate diet (6). Information on changes in body weight and composition of adult animals deprived of protein is of importance in predicting the effects of such dietary restriction in other species, including man, and in providing a basis for comparison of the effects of protein deprivation in gravid versus nongravid animals. The experiments reported here describe the effects of protein deprivation on body weight, voluntary feed consumption, serum protein level and body composition of adult nongravid female rats.

EXPERIMENTAL PROCEDURE

General. Female rats of the Carworth strain were used in two experiments. Rats were assigned randomly to dietary treatment at 12 to 14 weeks of age, at body weights averaging approximately 225 and 215 g, in experiments 1 and 2, respectively. In each experiment rats were kept in individual wire-bottom cages and offered water ad libitum from glass water bottles attached to the cage, and feed ad libitum from porcelain feed dishes designed for minimum feed wastage. Feed consumption and body weight were recorded every sec-

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TABLE 1

Composition of diets (exps. 1 and 2)

Diet	C	PF
	%	%
Glucose [1]	75.0	89.0
Corn oil [2]	3.0	3.0
Casein [3]	14.0	—
Minerals [4]	4.0	4.0
Vitamins [5]	4.0	4.0

[1] Cerelose, Corn Products Company, Argo, Ill.
[2] Mazola, Corn Products Company.
[3] Crude, 30-mesh, National Casein Company, New York.
[4] Jones, J. H., and C. Foster 1942 J. Nutr., 24: 245. (Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.)
[5] Vitamin diet fortification mixture in dextrose (Nutritional Biochemicals Corporation). Supplies per kilogram of diet: (in milligrams) α-tocopheryl acetate, 146; menadione, 65; thiamine·HCl, 29; riboflavin, 29; niacin, 129; Ca pantothenate, 88; pyridoxine·HCl, 29; inositol, 146; *p*-aminobenzoic acid, 146; folic acid, 0.3; biotin, 0.59; ascorbic acid, 1320; and choline chloride, 2.19 g; vitamin B_{12}, 40 μg; and vitamin A, 2633 IU and vitamin D_3, 293 IU.

ond day. The compositions of the protein-free (PF) and control (C) diets are shown in table 1. The data were treated statistically by analysis of variance (7).

Experiment 1. Forty-eight rats were assigned in groups of 12 to the following four dietary treatments: 1) casein diet (C) throughout the 26-day period; 2) protein-free diet (PF) throughout the 26-day period; 3) casein, days zero to 9; protein-free, days 10 to 26 (C-PF); and 4) protein-free, days zero to 9; casein, days 10 to 26 (PF-C). Total feed consumed by each rat during the periods day zero to 9 and 10 to 25 was recorded. Body weights were recorded every second day.

Experiment 2. Twenty-eight rats were assigned to the following two dietary treatments: 1) casein diet (C) throughout the experimental period, and 2) protein-free diet (PF) throughout the experimental period. The duration of the experiment was 23 days.

Feed consumption was recorded through day 22. At the end of the experiment all rats were killed with ether anesthesia. A blood sample was obtained from the abdominal aorta for total serum protein determination (8).

The entire body, including the gastrointestinal tract and contents was freeze-dried,[1] then ground through a Wiley mill with dry ice added in an equal quantity by weight to maintain a low temperature of the carcass lipids during grinding. The finely ground sample was then exposed at room temperature overnight to allow release of all carbon dioxide and was stored at 5° in an air-tight bottle until analyzed. Gross energy was determined by bomb calorimetry and protein by the Kjeldahl procedure. The experimental error introduced by including the contents of the gastrointestinal tract was considered to be less than would be introduced by an attempt to remove the contents. The contribution of the contents would presumably introduce a similar error for both groups with respect to energy and water, but would favor the control group with respect to protein. The contribution of the contents to the total composition was ignored in making the calculations.

RESULTS AND DISCUSSION

The results of experiments 1 and 2 are summarized in tables 2 through 5. The body weight of rats fed the protein-free (PF) diet showed an immediate reduction (significant, $P < 0.005$ day 2 and thereafter) and continued to decline throughout the experimental period in both experiments. The weight of rats fed the adequate diet (C) remained relatively constant throughout the same period. Percentage changes in weight for experiments 1 and 2 are summarized in table 2. In experiment 1, the weight loss of PF rats was 15.6% after 10 days, 27.6% after 20 days and 32.9% after 26 days. Corresponding values for experiment 2 were 13.6, 23.8 and 25.7%, respectively. These values are comparable to reported values for male rats at 20 days (6), but higher than those reported for dogs over a 50- to 60-day period (9). In rats fed the C diet for 9 days (exp. 1) and then transferred to the PF diet, weight loss was at approximately the same rate as that of rats fed the PF diet throughout as indicated by the parallel weight curves of these rats during the period of PF feeding. Conversely, in rats fed the PF diet for 9 days followed by the C diet, weight gain after transfer to the C diet was rapid and body weight was restored to the initial level within 8 days of refeeding.

Feed consumption by periods is summarized in table 3 for experiment 1. The

[1] Desivac, F. J. Stokes Corporation, Philadelphia, Penna.

TABLE 2

Percentage changes in body weight of adult female rats fed adequate or protein-free diets (exps. 1 and 2)

Experiment no.	Diet [1]	Initial	Body wt, % of initial wt		
			Day 10	Day 20	Day 26
1	PF	100	84.4	72.4	67.1
	C	100	102.2	98.7	103.1
2	PF	100	86.4	76.2	74.3 [2]
	C	100	106.1	110.3	110.7 [2]

[1] C = casein, PF = protein-free (see table 1).
[2] Twenty-three days.

TABLE 3

Feed consumption of adult female rats fed adequate or protein-free diets (exp. 1, g)

	Diet [1]			
	C–C	PF–PF	C–PF	PF–C
	g	*g*	*g*	*g*
Total feed intake per rat				
Period 1 [2]	200.3 ± 6.9 [3]	146.8 ± 6.4	192.9 ± 9.3	186.4 ± 11.6
Period 2	226.9 ± 12.9	185.8 ± 14.5	203.3 ± 11.3	284.2 ± 7.8
Combined	427.2 ± 18.4	320.1 ± 17.4	392.9 ± 16.5	470.5 ± 16.0

[1] C–C = casein throughout; PF–PF = protein-free throughout; C–PF = casein first period, protein-free second period; PF–C = protein-free first period, casein second period (see table 1).
[2] Period 1 = 12 days; period 2 = 14 days.
[3] Mean ± SD. Significant ($P < 0.005$) differences among diets (periods 1 and 2 combined).

striking feature is that total feed consumption per rat in the PF groups did not appear to be drastically reduced. Only in experiment 1 was feed consumption significantly ($P < 0.005$) reduced in rats fed the PF diet as compared with that in rats fed the C diet. In experiment 2 values were similar for rats fed the PF and C diets. It was impossible to completely prevent feed wastage. If it is accepted, however, that feed wastage accounted for only part of the recorded feed consumption figures, it must be concluded that a considerable amount of ingested energy was metabolized and excreted, probably as carbon dioxide and water, in the rats fed the PF diet. Increased heat increment in such animals has been demonstrated by body composition studies (10, 11).

The serum protein concentration of rats fed the PF diet for 23 days in experiment 2 was significantly ($P < 0.05$) reduced as compared with that of rats fed the C diet (6.09 versus 6.65 g/100 ml). The moderate rather than severe reduction was unexpected in view of the long depletion period. The body weight loss of PF rats (table 4) was probably accompanied by a loss in blood volume so that this moderate reduction in concentration of serum protein probably represents a large reduction in the absolute amount of serum protein. This relative insensitivity of total serum protein concentration to diet in the adult suggests that this parameter is a less valuable index of dietary protein nutrition in the adult than in the growing animal. Serum albumin and, to a lesser extent, total serum protein concentration is a sensitive criterion for assessing protein status in the growing animal (11).

TABLE 4

Body weight of adult female rats fed adequate or protein-free diets (exp. 2, g)

Day	Diet [1]	
	C	PF
0	214.5 ± 8.3 [2]	214.5 ± 14.5
9	230.5 ± 9.2	184.6 ± 14.0
16	235.8 ± 10.2	170.4 ± 14.2
23	237.2 ± 20.3	159.0 ± 11.8

[1] C = casein throughout; PF = protein-free throughout (see table 1).
[2] Mean ± SD. Significantly ($P < 0.01$) greater weight for rats fed diet C at days 9, 16 and 23.

Body composition of rats in experiment 2 fed the PF diet compared with that of rats fed the C diet suggests a large differ-

TABLE 5

Body composition of adult female rats fed adequate or protein-free diets (exp. 2)

	Diet [1]	
	C	PF
No. of rats	12	16
Avg initial wt, g	214.5	214.5
Avg final wt, g	236	169
Total body energy, kcal [2]	490.8	314.3
Total body protein, g [2]	46.8	35.9
Total body water, g [2]	151.2	109.5
Energy per gram dry wt, kcal [2]	5.789	5.235
Body composition, %		
Protein [2]	19.9	21.3
Water	64.1	64.9
Fat + carbohydrate + ash [3]	14.6	13.8

[1] C = casein throughout; PF = protein-free throughout (see table 1).
[2] Significant ($P < 0.005$) difference between diets.
[3] Significant ($P < 0.05$) difference between diets.

ence in heat increment and agrees with the data of Miller and Payne (10) in young rats and Pond et al. (11) in young pigs. The chemical composition of the weight loss was not measured directly but the final body composition of rats fed the PF and C diets was determined. A comparison of the protein and energy content of these rats is given in table 5. Total body energy, protein and water were less by an average of 176.5 kcal (36%), 10.9 g (23%) and 41.7 g (28%), respectively, in rats fed the PF diet than in those fed the C diet. The energy concentration expressed as kilocalories per gram dry weight was 10.6% greater in rats fed the C diet. All of the above differences were highly significant ($P < 0.005$).

Body composition expressed as percentage protein and water, by analysis and percentage other components (fat, carbohydrate and ash) by difference, revealed no effect of diet on water concentration, but a significantly higher percentage of protein ($P < 0.005$) and lower percentage of fat, carbohydrate and ash ($P < 0.05$) in rats fed the protein-free diet. This reflects a greater relative loss of body fat than of body protein associated with protein deprivation. Garrow (9) reported a greater percentage loss of nitrogen than of body weight (30% nitrogen loss versus 13 to 18% weight loss) in protein-deprived dogs and suggested that edema was responsible. The data from the present rat experiment fail to demonstrate edema, but protein depletion may have been less severe since the period of feeding was less than one-half as long.

Williams (6) reported a loss of 45% of initial body weight of male rats over a period of 80 days of protein deprivation. At this time they were emaciated and edematous, but responded quickly to protein repletion and were grossly identical to controls after 50 days of repletion. The adverse effects of protein deprivation were probably more severe in the experiment of Williams (6) than in the present one as the young adult male rats used in the former were still growing whereas the females in the latter were much closer to mature weight initially.

The present work demonstrates that the adult female rat can survive loss of at least one-third of body energy and one-fourth of body protein induced by protein deprivation without developing severe hypoproteinemia, emaciation or edema. The degree of depletion of these body nutrients compatible with survival and freedom from permanent damage is unknown but it is a subject of important concern.

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Effect of Dietary Amino Acid Composition on the Accumulation of Lipids in the Liver of Growing Rats

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ABSTRACT In an attempt to clarify the biochemical nature of the variances in the lipid content of liver of rats fed either low egg albumin or low casein diets, the effect of specific diets, consisting of amino acid mixtures which simulated egg albumin and casein and in which the level of S-containing amino acids varied, was examined. The decline in the level of S-containing amino acids (methionine and cystine) in the amino acid mixtures simulated egg albumin, causing a lowering of the liver lipids. Conversely, the high level of S-containing amino acids in the amino acid mixtures simulated casein, causing an elevation of the liver lipid content. Consequently, the dietary level of S-containing amino acids was considered to be the major factor governing the accumulation of liver lipids in rats fed the low protein diets. Our results indicate also that the ratio of methionine to cystine in the amino acid mixtures does not affect the lipid level in the liver.

An antilipotropic effect of methionine in rats fed threonine-deficient diets was reported by Harper et al. in 1954 (1). Later Yoshida and Ashida (2, 3) presented experimental evidence showing that accumulation of lipids in the livers of rats fed diets consisting of casein supplemented with methionine may be attributed to excess calories in relation to the retention of nitrogen.

It has been generally accepted that feeding low protein diets causes a pronounced accumulation of lipids in the rat liver. Feeding rats egg albumin (4), rice protein (5) or wheat gluten [2] as the sources of protein was shown to cause an accumulation of lipids in the liver. In the liver of rats fed low casein diets, however, the lipid production was not detected (1). In the work reported in this paper, we attempted to investigate the effect both of low casein and of low egg albumin diets on the prevention and enhancement of the lipid accumulation in rat liver.

EXPERIMENTAL

Male weanling rats of the Donryu strain were housed individually in a suspended wire-bottom cage at an environmental temperature of approximately 25°. Throughout the experimental periods, the light was switched off every 12 hours. Rats were fed experimental diets and water ad libitum for 14 days, except as otherwise indicated. Nitrogen sources of the diets used were: experiment 1, 5 and 8% egg albumin, and 7 and 11% casein; and in experiments 2 through 6, amino acid mixtures simulated casein or egg albumin. The amino acid composition of diets 2 through 6 are shown in table 1. All diets contained the following additional substances: (in percent) salt mixtures (6), 4; corn oil, 5; water-soluble vitamin mixtures in lactose (6), 0.85; choline chloride, 0.15; and sucrose to make each diet 100%. The content of vitamins A, D and E was 600 IU, 60 IU and 10 mg/100 g ration, respectively. The amount of food consumption was determined daily. At the end of the feeding period, rats were killed; the livers were removed immediately and weighed. They were then homogenized with deionized water using a Potter-Elvehjem type Teflon homogenizer. The resulting homogenate was dried and ground. Carcass materials excluding liver, stomach and intestines were frozen and minced. The lipid content in liver and carcass was determined by ether extraction using a Goldfish apparatus.

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[2] A. Yoshida, and K. Ashida, unpublished data.

TABLE 1

Compositions of amino acid mixtures that simulated 7% casein and 5% egg albumin

	Casein [1]	Egg albumin [1]
	% in diet	*% in diet*
L-Arginine·HCl	0.346	0.356
L-Histidine·HCl·H_2O	0.288	0.146
L-Isoleucine	0.460	0.324
L-Leucine	0.706	0.440
L-Lysine·HCl	0.703	0.374
DL-Methionine	0.217	0.195
L-Cystine	0.027	0.122
L-Phenylalanine	0.378	0.319
L-Tyrosine	0.410	0.208
L-Threonine	0.298	0.221
L-Tryptophan	0.094	0.076
L-Valine	0.519	0.390
L-Alanine	0.236	0.378
L-Asparagine	0.260	0.198
L-Aspartic acid	0.260	0.198
L-Glutamic acid	1.620	0.705
Glycine	0.140	0.187
L-Proline	0.826	0.189
L-Serine	0.466	0.393
$NaHCO_3$	0.63	0.41

[1] Calculated from Orr, M. L., and B. K. Watt 1957 Amino Acid Content of Foods.

RESULTS

In experiment 1, the effect of each diet, egg albumin and casein, on the liver lipid accumulation was examined at two levels. Comparing the biological values of egg albumin and casein, it was expected that rats fed 5 and 8% egg albumin diets might have a growth rate equal to those fed 7 and 11% casein diets, respectively. The body weight gain of rats in weeks 1 and 2 (table 2) shows that this prediction was attained by week 2. It should be noted, however, that due to unknown reasons the body weight of rats fed egg albumin decreased in week 1. Results show that both 5 and 8% egg albumin diets caused an elevation of the lipid content in the liver compared with the 7 and 11% casein diets.

Next, the liver lipid content in rats fed diets consisting of amino acid mixtures simulating 5% egg albumin and 7% casein, was determined (exp. 2); the results are shown in table 3. It appears that essentially a similar result as that served by the respective protein diets was obtained (cf. table 2).

Usually, low protein diets show a tendency to cause an elevation of the liver

TABLE 2

Content of liver lipids in rats fed low egg albumin and casein diets (exp. 1)

Diet	Gain in body wt		Liver wt per 100 g body wt	Liver lipids [1]
	Week 1	Week 2		
	g	*g*	*%*	*%*
5% egg albumin	−2.6 ± 0.8 [2]	2.1 ± 0.4	5.6 ± 0.2	23.9
8% egg albumin	3.4 ± 1.9	11.4 ± 1.6	5.9 ± 0.3	19.5
7% casein	2.9 ± 0.8	1.9 ± 1.2	5.0 ± 0.3	9.4
11% casein	12.7 ± 1.7	11.3 ± 2.1	4.8 ± 0.1	8.0

[1] Dry weight basis; obtained from pooled livers.
[2] Means ± SE of mean for five rats.

TABLE 3

Content of liver lipids in rats fed amino acid mixtures that simulated egg albumin and casein (exp. 2)

Diet	Food consumption	Gain in body wt	Liver wt per 100 g body wt	Liver lipids [1]
	g	*g*	*%*	*%*
Amino acid mixture that simulated 5% egg albumin	79 ± 4 [2]	9.2 ± 1.6	5.8 ± 0.1	22.2 ± 2.3
Amino acid mixture that simulated 7% casein	80 ± 5	8.2 ± 1.0	4.5 ± 0.2	8.8 ± 0.8

[1] Dry weight basis.
[2] Means ± SE of mean for five rats.

lipid content. Therefore, it appears that the effect of diets containing either 5% egg albumin, or an amino acid mixture that simulated 5% egg albumin, might be attributed to the lower nitrogen content, compared with diets containing 7% casein or an amino acid mixture that simulated 7% casein. To examine whether or not this was the case, the effect of an amino acid mixture that simulated either 7% egg albumin or 7% casein was studied (exp. 3). Analytical results of the liver lipid content are presented in table 4. Results clearly show the higher lipid level in the liver was caused by the egg albumin diet compared with the casein diet, indicating that the primary factor involved in lipid accumulation is the difference in amino acid composition, and not the level of protein content in the two diets.

One distinct difference is in the amino acid composition of egg albumin and casein, and in the amount of S-containing amino acids, methionine and cystine; 0.195% and 0.122%, respectively, for egg albumin, and 0.217% and 0.027%, respectively, for casein. Therefore, experiments were designed to determine whether the lower lipid content in the liver of rats fed casein diets was caused by the lower content of the S-containing amino acids (methionine plus cystine) or by the higher ratio of methionine to cystine compared with the effects of egg albumin (exps. 4 and 5). In experiment 4, the effect of methionine and cystine was examined by supplementing these two to the amino acid mixture that simulated 7% casein at the different levels. The ratio of methionine to cystine was maintained at the same level as that in casein. Results presented in table 5 clearly show that the addition of higher amounts of methionine and cystine caused elevation of liver lipids (last column). The content of carcass lipids also slightly increased under this condition. Essentially similar results were obtained in experiment 5, in which the

TABLE 4

Content of liver lipids in rats fed amino acid mixtures that simulated egg albumin and casein (exp. 3)

Diet	Food consumption	Gain in body wt	Liver wt per 100 g body wt	Liver lipids [1]
	g	*g*	%	%
Amino acid mixture that simulated 7% egg albumin	126 ± 2 [2]	31.4 ± 2.4	5.5 ± 0.2	27.5 ± 3.0
Amino acid mixture that simulated 7% casein	90 ± 4	11.3 ± 2.0	5.1 ± 0.2	10.2 ± 0.8

[1] Dry weight basis.
[2] Means ± SE of mean for five rats.

TABLE 5

Effect of methionine and cystine supplement to amino acid mixture that simulated 7% casein at the same ratio as that in casein on the lipid content in liver and carcass (exp. 4)

Content of S-containing amino acids in diet			Food consumption	Gain in body wt	Liver wt per 100 g body wt	Liver lipids [1]	Carcass lipids [2]
DL-Methionine	L-Cystine	Sum					
%			*g*	*g*	*g*	%	%
0.217	0.027	0.244 [3]	99 ± 2 [4]	8.6 ± 0.3	5.2 ± 0.2	8.9 ± 0.4	13.1
0.250	0.031	0.281	101 ± 1	13.3 ± 2.0	5.4 ± 0.1	8.8 ± 0.5	12.8
0.282	0.035	0.317	108 ± 4	19.6 ± 1.5	5.7 ± 0.2	9.1 ± 0.6	13.5
0.382	0.047	0.429 [5]	123 ± 8	25.0 ± 0.3	6.2 ± 0.3	22.6 ± 1.0	16.4

[1] Dry weight basis.
[2] Wet weight basis; obtained from the pooled carcass.
[3] Content of methionine and cystine is the same as that in 7% casein.
[4] Means ± SE of mean for five rats.
[5] Ratio of sulfur-containing amino acids to threonine is the same as that in egg albumin.

TABLE 6

Effect of methionine and cystine supplement to amino acid mixture that simulated 7% casein at the same ratio as that in egg albumin on the lipid content in liver and carcass of rats (exp. 5)

Content of S-containing amino acids in diet			Food consumption	Gain in body wt	Liver wt per 100 g body wt	Liver lipids [1]	Carcass lipids [2]
DL-Methionine	L-Cystine	Sum					
%			g	g	g	%	%
0.150	0.094	0.244 [3]	113 ± 4 [4]	7.1 ± 1.3	5.2 ± 0.2	10.7 ± 0.2	10.7
0.173	0.108	0.281	121 ± 6	12.8 ± 2.8	5.3 ± 0.1	12.6 ± 1.5	12.3
0.195	0.122	0.317	131 ± 4	22.8 ± 2.2	5.1 ± 0.2	13.1 ± 1.4	14.8
0.264	0.165	0.429	133 ± 10	22.6 ± 3.5	6.1 ± 0.1	23.1 ± 2.7	14.3

[1] Dry weight basis.
[2] Wet weight basis; obtained from the pooled carcass.
[3] Content of methionine and cystine is the same as that in 7% casein.
[4] Means ± SE of mean for five rats.

TABLE 7

Effect of reduction of methionine and cystine content in amino acid mixture that simulated 5% egg albumin on the lipid content in liver and carcass (exp. 6)

Content of S-containing amino acids in diet			Food consumption	Gain in body wt	Liver wt per 100 g body wt	Liver lipids [1]	Carcass lipids [2]
DL-Methionine	L-Cystine	Sum					
%			g	g	g	%	%
0.195	0.122	0.317 [3]	103 ± 3 [4]	11.6 ± 2.0	6.5 ± 0.2	21.4 ± 2.3	15.6
0.154	0.096	0.250	97 ± 4	10.3 ± 1.6	5.9 ± 0.2	13.6 ± 3.3	15.6
0.123	0.077	0.200	99 ± 6	9.1 ± 2.5	5.4 ± 0.2	11.1 ± 0.9	16.0

[1] Dry weight basis.
[2] Wet weight basis; obtained from the pooled carcass.
[3] Content of methionine and cystine is the same as that in 5% egg albumin.
[4] Means ± SE of mean for five rats.

different amounts of methionine and cystine added to the amino acid mixture simulated 7% casein, the ratio being similar to that in egg albumin (table 6). From these experiments, therefore, it appears certain that the low level of lipids in the liver of rats fed low casein diets is not related to the ratio of methionine to cystine, but rather related to the amounts of S-containing amino acids in the diet. Indeed, as shown in experiment 6, the reduction of the percentage of S-containing amino acids (methionine and cystine) in the amino acid mixture simulated 5% egg albumin, causing a decline of the liver lipid content (table 7). However, S-amino acids had little effect on the content of carcass lipids. Overall findings strongly suggest that the elevation of the lipid content in the livers of rats fed an amino acid mixture that simulated 5% egg albumin is related to the amount of S-containing amino acids (methionine and cystine) in the diet.

DISCUSSION

Although egg albumin shows the highest biological value among natural proteins, our present experiments have demonstrated that feeding rats diets containing 5% egg albumin or amino acid mixtures that simulated 5% egg albumin, caused a marked elevation of the liver lipids (tables 2 and 3). On the other hand, it was found that reduction of the S-containing amino acids (methionine and cystine) in the diet consisting of the amino acid mixtures that simulated 5% egg albumin did not promote lipid accumulation in the liver (table 7). Furthermore, increased amounts of S-containing amino acids in the amino acid mixture that simulated 7% casein resulted in the elevation of the liver lipids (tables 5 and 6). It will be recalled that the accumulation of lipids in the liver occurs by feeding rats diets containing 7 to 9% casein supplemented with 0.3% methionine or cystine (1). These findings strongly

indicate that one of the major factors governing the level of liver lipids will be the content of S-containing amino acids in the diet. Thus, our results can be interpretable in terms of the amino acid imbalance.

The toxic effect of excessive methionine in the dietary composition has been reported (7, 8). The maximum amount of methionine plus cystine in diets used in the present investigation was 0.429%, which is far from the level which may bring about growth retardation.

In connection with our present study, the following reports deserve our attention. Williams et al. (9) reported the changes in the liver lipid content in rats fed the protein-free diet supplemented with individual amino acids. They found that methionine, cystine and, to a lesser extent, valine exhibited an antilipotropic effect. Both leucine and phenylalanine, however, depressed the level of liver lipids. Other essential amino acids did not affect the lipid content to any extent. Charkey and Kano (10) and Charkey and Hougham (11) demonstrated an antithyrotoxic effect of methionine by determining the changes of the content of glutathione in chick plasma. Thus, it appears that both antilipotropic and antithyrotoxic effects of S-amino acids might be reflected in the experimental findings reported in this paper. The nature of these closely related phenomena at the enzymatic level provides a basis for future investigation.

Finally, Tucker and Eckstein (12) reported that a supplement of 0.5% methionine to a choline-deficient diet containing 15% casein and 40% lard effectively reduced the liver lipid content of rats. They found, however, that a supplement of 0.5% cystine to the diet elevated the level of liver lipids. In their study methionine and cystine exhibited apparently opposite effects on the formation of liver lipids. As shown in our study (tables 5 and 6), the changes in the ratio of methionine to cystine in the diets containing choline do not demonstrate a marked difference in the liver lipid level. It is not clear at this time why the results of our investigation differ from those of Tucker and Eckstein.

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Effect of Dietary Magnesium on the Development of Nephrocalcinosis in Rats [1]

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ABSTRACT Weanling rats fed a semipurified diet containing 0.4% Ca, 0.56% P and 0.05% Mg rapidly developed a nephrocalcinosis in which deposits of calcium-phosphate mineral formed in the region of the corticomedullary junction of the kidney. Calcification was more severe in female rats than in male rats. Because 0.05% Mg is considered an adequate dietary level, and because both serum and kidney content of magnesium were normal, the calcification in the kidneys was not likely to have developed as a result of a simple dietary magnesium deficiency. However, raising the dietary magnesium to 0.45% with either magnesium chloride or magnesium carbonate increased serum magnesium and prevented the development of nephrocalcinosis. The protective action of magnesium could not be explained by alterations in the concentrations of calcium and phosphorus in the serum. When the supplemental magnesium was provided as carbonate, renal and bladder calculi composed principally of magnesium and phosphorus formed quickly, probably because of a high concentration of magnesium in an alkaline urine.

Rats of different strains raised at the University of Otago (black, white and hooded, and also a Sprague-Dawley strain recently imported from the United States) developed a nephrocalcinosis in which stainable deposits, shown by analysis to be calcium phosphate mineral, formed at the corticomedullary junction of the kidneys. The condition was first noted by Cousins and Geary (1) only in female rats of the stock colony fed the stock pelleted diet or a semipurified diet used in our laboratory for studies on trace elements and dental caries. We have found it in male rats, but whereas every female fed either of these diets is affected, the incidence and severity are more variable in males, and the semipurified diet caused more calcification than the pelleted diet. We have confirmed the finding of Cousins and Geary that the deposits are present in most rats by the time they are 6 weeks old. We have even seen them in 4-week-old rats after only 1 week on the semipurified diet. Alterations to the mineral composition of the diet, to raise the Ca/P ratio from 0.7 to 1.2 or to change the type of calcium and phosphate salts used, did not affect the incidence of the condition.

The nephrocalcinosis is similar in appearance to that occurring in magnesium deficiency; and although by the usual standards (2) our diets contain adequate magnesium (0.05%) we decided to investigate the effect of increasing the magnesium content of the diet on the development of nephrocalcinosis. In trial experiments we observed that when the magnesium content of the semipurified diet was increased from 0.05 to 0.09% with magnesium chloride the calcification was reduced but not eliminated; also, that when dietary magnesium was increased to 0.45% with magnesium carbonate, the results differed in rats of different ages. Female rats started on this regimen at 6 weeks of age continued to develop nephrocalcinosis, but if started at 21 days of age, the animals quickly formed numerous renal and bladder stones composed mainly of magnesium and phosphate. Because these might have been caused by a high concentration of magnesium in an alkaline urine, we compared the effects of giving the extra magnesium as carbonate and as chloride, which should have made the urine acid.

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EXPERIMENTAL

Animals. Rats of Wistar origin were maintained as a closed random-bred colony for nearly 40 years; they were raised on CS40 diet,[3] weaned at 21 days of age and housed individually in screen-bottom stainless steel cages. Food and distilled water were available ad libitum.

Procedures. Sixty weanling rats were divided into three experimental groups, each comprising 10 males and 10 females of similar average weight (42 to 43 g). Group A received the basal semipurified diet, CS40; group B, CS40 plus 0.4% Mg as $MgCO_3$; and group C, CS40 plus 0.4% Mg as magnesium chloride. The food and water intakes were measured daily and the animals weighed weekly. Many rats in group B were killed when it became apparent that their bladders were blocked by stones, and some rats died. Those rats which did not develop blockage of the bladder were killed at 8 weeks of age, after 5 weeks feeding of the diet. All rats killed before the end of the experiment were excluded from the tables of results. When the rats were killed, blood was collected by cardiac puncture under ether anesthesia. Serum was separated by centrifugation and analyzed for calcium, phosphorus and magnesium. The kidneys and urinary tracts of all animals were examined for mineral deposits, and a half kidney from each was fixed in buffered formol saline for histological examination. The remaining kidney tissue was dried at 110°; it was ashed in platinum crucibles at 500° overnight and the ash was taken up in 3 ml of 50% HCl. The solution was heated on a water bath to convert pyrophosphate to orthophosphate. Stones were analyzed for calcium, phosphorus, magnesium and ammonia nitrogen.

Analytical methods. Calcium was estimated by titration using EDTA and Cal-red indicator (3). Phosphorus was estimated by a colorimetric method (4) and magnesium was estimated by atomic absorption spectrophotometry.[4] Ammonia nitrogen was estimated by the method of Fawcett and Scott (5).

RESULTS

Weight gains, food and water intakes (table 1). All rats in groups A and C survived and all grew well, but mortality was high in group B because many rats developed urethral blockage after only a few days on the diet and died or had to be killed. Only four males and one female survived to the end of the experiment.

Urinary pH. The reaction of a fresh sample of urine from each rat was tested, using fine range indicator paper, just before the rats were killed. The mean pH values were: 6.7, 7.3 and 6.7 for groups A, B and C, respectively.

Serum analyses. Concentrations of serum calcium were similar in females of all groups and in males of all groups, but males tended to have a higher mean serum calcium than females (table 1). Serum phosphorus levels were slightly higher in rats fed high magnesium diets B and C than in rats fed basal diet A. Serum magnesium was significantly raised on high magnesium diets B and C ($P > 0.001$).

Animals in group B killed because of bladder blockage had quite abnormal serum levels of calcium, phosphorus and magnesium, e.g., one male rat killed after 14 days on diet B had serum calcium 8.8, serum phosphorus 21.8 and serum magnesium 10.7 mg/100 ml. All these rats had raised serum magnesium and serum phosphorus while calcium was lowered in some of them. It appeared that serum magnesium rose before serum phosphorus since rats with moderately increased magnesium had nearly normal phosphorus, whereas rats with high serum magnesium always had high serum phosphorus and most had lowered serum calcium.

Composition of the kidneys. Normal uncalcified kidney tissue contained approximately 0.3 mg Ca, 13.0 mg P and

[3] The semipurified diet (CS40) contained: (in percent) casein, 25; soybean oil, 3; vitamin mixture, 1.2; sucrose, 67.8; and 3% of a salt mixture containing: (in percent) NaH_2PO_4, 24.5; $Ca_3(PO_4)_2$, 34.0; KCl, 13.2; $MgSO_4$, 8.3; ferric ammonium citrate, 1.0; $MnSO_4 \cdot 4H_2O$, 0.7; $CuSO_4$, 0.09; $ZnCO_3$, 0.07; KIO_3, 0.01; and starch 18.13. The vitamin mixture provided the following ingredients per kilogram diet: (in grams) ascorbic acid, 1.25; choline chloride, 1.25; p-aminobenzoic acid, 0.375; and inositol, 0.125; (in milligrams) nicotinic acid, 32; Ca pantothenate, 25; thiamine·HCl, 15; riboflavin, 9.4; pyridoxine·HCl, 6.25; folic acid, 1.25; and alpha-tocopheryl acetate, 125; (in micrograms) biotin, 250; and vitamin B_{12}, 62.5; (in International Units) vitamin A acetate, 10,000; and vitamin D_3, 4,800. By analysis this basal diet contained 0.4% Ca, 0.56% P and 0.05% Mg. The supplements of $MgCO_3$ and $MgCl_2 \cdot 6H_2O$ were given at the expense of sucrose.

[4] Atomic absorption spectrophotometer, Hilger and Watts, Ltd, England, and Techtron Pty Ltd., Australia.

TABLE 1

Effect of magnesium content of the diet on weight gain, food and water intakes and mineral composition of the blood of rats

	Group A, CS40 (0.05% Mg)		Group B, CS40 + 0.4% Mg as $MgCO_3$		Group C, CS40 + 0.4% Mg as $MgCl_2 \cdot 6H_2O$	
	Males	Females	Males	Females	Males	Females
No. of rats	10	10	4	1	10	10
Food intake, g	483	405	424	400	461	381
Water intake, ml	533	445	545	565	738	592
Wt gain, g [1]	181.8 ± 29.9 [2]	113.2 ± 17.5	152.0	112.0	162.3 ± 17.9	107.9 ± 14.1
Serum calcium, mg/100 ml	11.0 ± 0.52	10.4 ± 0.24	11.1	10.7	10.9 ± 0.37	10.2 ± 0.35
Serum phosphorus, mg/100 ml	8.5 ± 1.09	7.5 ± 1.01	9.7	8.8	9.4 ± 0.80	8.0 ± 0.93
Serum magnesium, mg/100 ml	1.99 ± 0.13	2.01 ± 0.13	3.10	3.16	3.18 ± 0.42	3.10 ± 0.72

[1] Weight gain from 21 to 56 days of age.
[2] Means ± SD.

TABLE 2

Effect of adding magnesium to diet CS40 on the composition of the kidney

	Group A, CS40 (0.05% Mg)		Group B, CS40 + 0.4% Mg as $MgCO_3$		Group C, CS40 + 0.4% Mg as $MgCl_2 \cdot 6H_2O$	
	Males	Females	Males	Females	Males	Females
No. of rats	10	10	4	1	10	10
Wet wt kidneys, g	1.88 ± 0.25 [1]	1.71 ± 0.29	1.55	1.34	1.85 ± 0.24	1.45 ± 0.15
Dry weight, as % wet wt	25.4 ± 0.71	24.9 ± 0.90	24.7	25.5	25.6 ± 1.10	25.0 ± 0.69
Ash wt, as % dry wt	5.73 ± 0.15	12.49 ± 3.5	5.72	5.94	5.53 ± 0.23	5.51 ± 0.20
Kidney calcium [2]	0.81 ± 0.48	26.49 ± 13.3	0.43	0.44	0.30 ± 0.02	0.31 ± 0.02
Kidney phosphorus [2]	13.07 ± 0.82	27.32 ± 6.35	13.94	14.5	13.14 ± 0.72	13.81 ± 0.41
Kidney magnesium [2]	0.94 ± 0.05	1.53 ± 0.29	1.11	1.06	0.91 ± 0.03	0.90 ± 0.03
Calcium phosphate nephrocalcinosis	+	+	0	0	0	0
Magnesium phosphate calculi	0	0	+	+	0	0

[1] Mean ± SD.
[2] Milligrams per gram dry kidney.

0.9 mg Mg/g dry tissue. The phosphorus content was much more variable than either the calcium or magnesium of uncalcified tissue, and in our rats varied from 11 to 14 mg/g dry tissue. In group A all animals had typical calcium phosphate nephrocalcinosis which was much more severe in females than in males as can be seen from the increased ash content and raised levels of calcium, phosphorus and magnesium of the kidneys (table 2). No renal or bladder stones were observed in

groups A or C; this was as expected for no renal or bladder stones had ever been seen with this form of nephrocalcinosis in our rats.

Surviving rats in group B did not show calcium deposits in the tubules, but they had magnesium phosphate calculi in their kidneys and urinary tracts. A pooled sample made up of calculi from all males in the group contained 2.1% Ca, 17.9% P, 14.7% Mg and 0.4% ammonia nitrogen, while a sample of pooled calculi from all females contained 0.5% Ca, 15.8% P, 12.2% Mg and 2.2% ammonia nitrogen. The calcium, phosphorus and magnesium contents of the kidneys were slightly higher than those in uncalcified kidney tissue. Kidneys from rats killed because of bladder blockage had a higher magnesium and phosphorus content than surviving rats. All rats of group B had numerous calculi in the urinary tract, in some cases with dilatation of the ureters and renal pelvis presumably caused by back pressure.

Animals in group C had no nephrocalcinosis or calculi, and the calcium, phosphorus and magnesium content of the kidney was normal. The magnesium level was slightly higher than found by many workers, but this is probably attributable to the high dietary magnesium; Forbes (6) fed a diet containing 0.1% Mg and found a similar amount of magnesium in the kidney tissue of rats.

DISCUSSION

Food intakes were similar and the weight gains of all groups were good. The increased water intake of rats in group C was probably related to the loss caused by their severe diarrhea, for it was accompanied by only a small increase in volume of urine. Thus, the urine output was approximately 12 ml/day in group C; this did not seem sufficiently higher than the 9 ml produced daily by rats in group A to explain the absence of calcification by dilution of the urine.

Rats in group B given magnesium carbonate produced an alkaline urine. Rats in groups A and C had a similar urinary pH, but this did not mean that the hydrogen ion excretion in the urine was similar. We found in other experiments [5] that when rats were fed an acidifying diet (CS40 containing 2.7% ammonium chloride) the urinary pH fell for only a few hours and then rose to a similar level to that found in urine of rats fed the basic CS40 diet. This was due to an increased excretion of hydrogen ions as ammonium by rats fed the acidifying diet. Because rats developed nephrocalcinosis when fed this high level of ammonium chloride, which acidified the urine more than the magnesium chloride diet used in the experiment reported here, the absence of nephrocalcinosis in animals of group C cannot be attributed to acidification of the urine by magnesium chloride.

Magnesium deficiency has repeatedly been shown to cause nephrocalcinosis, whereas high dietary intakes of magnesium have been shown to reduce kidney calcification in rats. Thus, Cunningham (7) reduced stone formation by raising the dietary magnesium, and concluded that a dietary imbalance of calcium, phosphorus and magnesium caused stones to be formed. Gershoff and Andrus (8, 9) were able to prevent the formation of renal deposits of calcium oxalate by feeding 0.4% Mg in the diet. Selye (10) demonstrated a protective effect of magnesium by treating rats with magnesium at the same time he administered doses of parathyroid hormone which otherwise caused renal calcification. The magnesium content of the CS40 diet met the recommended level, and rats in group A had both a normal serum magnesium concentration and a normal kidney magnesium content. As mentioned in the introduction, increasing magnesium from 0.05% to 0.09% did not eliminate nephrocalcinosis and our rats could, therefore, hardly have developed nephrocalcinosis as a result of a simple magnesium deficiency. Increasing the magnesium content of the diet to 0.45%, however, which significantly raised the serum magnesium concentration, prevented the development of nephrocalcinosis. The protective action of large dietary supplements of magnesium did not appear to be explicable by changes in the concentrations of calcium and phosphorus in the blood. These were normal in the serum of rats of group A. Increasing the dietary magnesium did not affect serum calcium,

[5] Unpublished observations.

but slightly increased serum phosphorus (groups B and C, table 1). In vitro, magnesium has been shown to increase the solubility of both calcium oxalate and calcium phosphate (11, 12). Moreover, high dietary magnesium increases citrate excretion (8, 9) and this would tend to solubilize calcium salts within the renal tubules. Mukai and Howard (13) showed that the ability of urine from stone formers to calcify rachitic cartilage was related to the magnesium content of the urine; addition of magnesium to calcifying urine rendered it noncalcifying; removal of magnesium from noncalcifying urine caused it to calcify cartilage. Apart from such physicochemical interactions, calcium and magnesium may compete for reabsorption in the renal tubule and for absorption from the gut (14), so that an increased amount of one ion should decrease the absorption of the other. Evans et al. (15), in a study of magnesium excretion in normal and stone-forming patients, showed that the mean daily urinary magnesium excretion of "normocalciuric" renal-stone formers was significantly lower than that of the normocalciuric control group. They suggested that a low urinary magnesium excretion might be a factor in some cases. Several reports have been made on the beneficial use of magnesium oxide to prevent the recurrence of renal calculi in human patients[6] (16). There is also some evidence that raising serum magnesium may reduce secretion of parathyroid hormone (17, 18). In our rats, high dietary magnesium raised the concentration of magnesium in the serum and prevented the formation of calcium-phosphate mineral deposits in the kidneys. This could have been due to a change in the solvent character of the tubular fluid, to a reduction in secretion of parathyroid hormone, or to competition between calcium and magnesium ions in the renal tubules. The effect is not restricted to a particular diet, for we have also found that raising the magnesium content of the stock pellet diet from 0.25 to 0.9% prevented nephrocalcinosis in weanling rats.[7] It appears that once calcium-phosphate deposits are present in the kidney tubules, however, high dietary magnesium does not prevent further deposition.

There is evidence that under some experimental conditions hypermagnesemia and excess dietary magnesium may actually induce renal mineralization, but the mineral deposited then tends to be rich in magnesium. Watchorn (19) drew attention to the formation of renal calculi composed of 25.8% Ca, 10.3% P, 16.6% Mg and 0.1% N, in rats fed a diet containing 0.4% Ca, 0.5% P and 1.6% Mg. Earlier, Haag and Palmer (20) had noted formation of calculi in some rats fed low calcium, high magnesium and high phosphorus diets. Vermeulen et al. (21) found that when rats were fed a diet containing 0.44% Ca, 0.25% P and 0.2% Mg they developed magnesium ammonium phosphate calculi in the bladder but that changing the magnesium content of the diet to 0.07% lowered the concentration of magnesium in the urine and prevented the formation of calculus. Robbins et al. (22) produced calculi composed of 1.5% Ca, 15.9% P and 10.5% Mg by feeding rats a diet containing 0.3% Ca, 1.2% P, 0.1% K, 0.81% Na and 0.5% Mg. Reducing the magnesium content to 0.05%, or altering the mineral composition of this diet in a number of other ways, prevented urolithiasis, and Robbins stressed the importance of the mineral interrelationships of the diet in the development of urolithiasis. When 0.45% Mg was fed to our weanling rats with the 0.4% supplemental magnesium supplied as magnesium carbonate, renal and bladder stones formed within a few days, probably as a result of precipitation from an alkaline urine since no stones formed when the same amount of supplementary magnesium was given as magnesium chloride. Older rats which had nephrocalcinosis did not develop stones, however, and age may therefore be a factor in the development of magnesium phosphate stones on this diet.

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[6] Sauberlich, H. E., G. E. Bunce, C. A. Moore and O. G. Stonington 1964 Oral magnesium administration in the treatment of renal calculus formation. Amer. J. Clin. Nutr., *14*: 240 (abstract).

[7] See footnote 5.

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Influence of Age and Calcium-free Diet on Thyroparathyroidectomized Sheep [1,2]

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ABSTRACT Results of thyroparathyroidectomy on ruminants have been confounded by diet, age, sex, pregnancy, surgical procedure and postsurgical treatment. Two trials were conducted to study the effect of age and calcium-free diet on thyroparathyroidectomized sheep. The rate of decline in serum calcium was rapid in young thyroparathyroidectomized sheep and often followed by fatal tetany, even when the animal was maintained on a normal diet. Positive calcium balance observed in the young thyroparathyroidectomized sheep indicated no appreciable effect on calcium absorption. In contrast, adult sheep were more tolerant to thyroparathyroidectomy, being capable of correcting hypocalcemia and maintaining serum calcium levels above symptomatic levels even after extended time on a calcium-free diet. In these trials neither thyroparathyroidectomy nor feeding a calcium-free diet affected serum magnesium. Serum magnesium decreased only when feed intake decreased.

Although thyroparathyroidectomy was performed on ruminants as early as 1907 (1) and has subsequently been reported on a number of occasions (2–7), results have been confounded by diet, age, sex, pregnancy, surgical procedure and postsurgical treatment.

Smith et al. (3) observed a decline in serum calcium following parathyroidectomy in young goats which were between 8 and 9 months of age. Tetany was not observed until the goats were placed on low calcium diets. Stott and Smith (4) thyroparathyroidectomized eight calves ranging in age from 8 to 99 days and observed fatal tetany within 9 days in seven of their animals. The thyroparathyroidectomized adult cows, however, showed asymptomatic hypocalcemia, which was followed by a return to normal calcium levels without treatment. Mayer et al. (7) reported similar effects after total parathyroidectomy in adult cows. Payne and Chamings (6) thyroparathyroidectomized 32 goats and observed no difference in response between adult males and kids; tetany was rare in both.

METHODS

Two trials were conducted to study the effect of age and a calcium-free diet on thyroparathyroidectomized sheep.

Trial 1. Three adult sheep, about 18 months of age, were thyroparathyroidectomized, while three sham-operated sheep of the same age served as controls. All animals were placed in metabolism stalls and each was fed 907 g/day of a purified diet (table 1) for 2 weeks prior and subsequent to surgery, after which they were fed 907 g/day of the purified calcium-free diet shown in table 1. Daily feces and urine samples were collected and pooled during 7-day periods. Aliquots of the pooled samples were dried, weighed and ashed. The ash was dissolved in 4 N HCl and analyzed for calcium and magnesium by atomic absorption spectrophotometry.

The following surgical procedure was performed on all animals: Food was withheld for 24 hours and water for 12 hours prior to surgery. All animals received intravenously 1 mg/kg body weight of promazine hydrochloride as a preanesthetic sedative and anesthesia was induced 10 minutes later by the intravenous adminis-

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TABLE 1

Composition of purified diets

Ingredient	Basal diet	Calcium-free diet
	%	%
Cornstarch	34.37	34.37
Glucose [1]	24.37	24.37
Cellulose [2]	31.05	31.57
Urea [3]	4.20	4.20
Corn oil [4]	1.00	1.00
Choline chloride	0.10	0.10
Vitamins A and D [5]	0.02	0.02
K_2CO_3	2.21	1.32
K_2HPO_4	—	1.69
$CaHPO_4$	1.32	—
$MgSO_4$	0.12	0.12
$MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$	0.27	0.27
Na_2SO_4	0.25	0.25
NaCl	0.62	0.62
Trace minerals [6]	0.10	0.10

[1] Cerelose, Corn Products Company, Argo, Ill.
[2] Solka-Floc, B-W20, Brown Company, Berlin, N. H.
[3] Crystalline urea. Courtesy John Deere Chemical Company, Pryor, Okla.
[4] Mazola, Corn Products Company. Santoquin added to give 0.0125% in total diet.
[5] Containing 20,000 IU and 2,500 USP U/g vitamins A and D, respectively. Courtesy NOPCO Chemical Company, Harrison, N. J.
[6] Composition of the trace mineral mixture: (in milligrams/100 g diet) $FeSO_4$, 42.51; $MnSO_4 \cdot H_2O$, 15.37; $Na_2B_4O_7$, 12.56; $ZnSO_4 \cdot 7H_2O$, 26.35; $CuCO_3 \cdot Cu(OH)_2$, 1.97; KI, 0.07; $CoCl_2 \cdot 6H_2O$, 0.05; CaF_2, 0.20; $Na_2MoO_4 \cdot 2H_2O$, 0.50; $Cr_2(SO_4)_3$, 0.04; and Na_2SeO_4, 0.02.

tration of a 2.5% solution of thiamylal [6] sodium at a rate of approximately 13 mg/kg. Following endotracheal intubation anesthesia was maintained with a halothane [7]-oxygen mixture in a semiclosed circuit.

The approach to the external parathyroid gland was made with the animal in lateral recumbency. A skin incision was made from a point 2 cm below the base of the ear and 1 cm behind the vertical ramus, extending downward and backward for approximately 5 cm and terminating just above the jugular furrow. Subcutaneous tissues were separated by blunt dissection, great care being taken to prevent hemorrhage. The external jugular vein was located, and the occipital vein was identified, ligated and sectioned. Further blunt dissection between the external jugular vein and the brachiocephalic muscle revealed the carotid sheath, the external parathyroid gland usually being located in the adipose tissue just dorsal to the carotid sheath. The gland, which is spherical in outline, dark pink in color, and approximately 3 mm in diameter, was isolated by blunt dissection and the blood supply occluded by application of a hemostat. Subcutaneous tissues were closed with chromic gut and the skin with braided polyester sutures. The opposite external parathyroid gland was removed in a similar manner. Frozen sections of the isolated parathyroid glands were cut and stained with hematoxylin and eosin thereby permitting histological identification during surgery.

After removal of the external parathyroids the animal was then placed in dorsal recumbency and a midline incision made on the ventral aspect of the neck, extending posteriorly for 5 cm from a point just behind the cricoid cartilage. The subcutaneous tissues were separated by blunt dissection to expose the thyroid glands, which lie on either side of the trachea, extending from the 2nd to the 6th tracheal rings and having a connecting isthmus at the level of the 5th ring. Thyroid arteries were ligated as they entered the anterior poles of the glands and the thyroid glands, including the associated internal parathyroid glands, were excised. No attempt was made to identify the internal parathyroid glands.

Thyroid and parathyroid glands of sham-operated animals were similarly exposed but were left intact. In all cases postanesthetic recovery was rapid and healing was by primary union without complication.

Trial 2. Five sheep, about 6 months old, were thyroparathyroidectomized, while five sham-operated animals of the same age served as controls. Methods and procedures were identical to those described in trial 1.

RESULTS

Table 2 shows the values for serum calcium and magnesium in the adult sheep (trial 1) during the postoperative period. Sheep A1 was completely thyroparathyroidectomized and had a precipitous decrease in serum calcium during the 2 weeks following surgery. This period was complicated by inappetance which possibly contributed to the decrease in serum calcium (8). Upon return of appetite serum calcium values increased from 4.9 mg/

[6] Surital sodium, Parke Davis & Company, Detroit, Mich.
[7] Fluothane, Ayerst Laboratories, New York, N. Y.

TABLE 2

Effect of thyroparathyroidectomy on serum calcium and magnesium in adult sheep

Post surgery	TPX						Sham					
	A1		A2		A3		B1		B2		B3	
	Calcium [1]	Magnesium [1]	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium
days												
0	11.1	3.0	12.0	3.0	11.0	3.2	10.5	3.0	11.1	3.8	10.8	2.8
1	9.9	2.8	9.7	2.7	11.1	3.2	10.1	3.1	10.2	3.4	10.0	3.1
2	9.3	2.9	9.3	2.6	10.2	3.2	11.2	3.2	10.7	3.4	10.4	3.2
3	6.9	2.6	9.5	2.6	10.6	3.5	11.1	3.5	12.2	3.7	11.2	3.0
4	8.4	2.8	9.8	2.5	11.0	3.7	11.9	3.2	11.6	3.6	10.9	3.3
5	8.4	2.8	9.5	2.5	11.5	3.6	11.4	3.0	11.4	3.4	11.9	2.9
6	7.5	2.3	9.4	2.3	10.6	3.3	11.8	3.3	11.0	3.2	11.5	3.4
7	7.2	2.3	10.0	2.7	10.1	3.9	11.5	2.9	11.4	3.8	10.6	2.8
8	6.3	1.6	10.2	2.3	11.1	3.5	11.3	3.0	11.9	3.2	11.4	2.8
9	5.8	1.5	10.0	2.3	11.7	3.5	11.4	3.4	11.7	3.4	11.3	2.5
10	6.0	1.3	10.4	2.5	11.8	3.4	11.1	3.1	11.1	3.2	8.8	2.9
11	4.9	1.4	9.7	2.4	9.8	3.7	11.1	2.9	11.9	3.5	11.2	2.8
12	5.7	1.5	9.7	2.4	11.6	3.7	9.8	2.9	11.3	3.3	11.2	2.7
13	4.2	1.7	8.8	2.6	10.6	3.5	10.1	3.0	11.5	3.4	11.0	2.8
14 [2]	4.9	2.1	8.6	2.6	10.2	3.4	11.4	2.9	10.3		10.6	2.9
15	5.0	2.1	9.7	2.5	9.9	3.2	9.9	3.1	10.1		9.3	2.9
16	4.7	2.1	9.3	2.6	9.4	3.4	9.8	3.3	10.7		10.6	2.9
17	5.0	2.2	9.8	2.9	9.2	3.3			10.3		10.4	
18							10.2	3.2			10.7	3.1
19	5.9	2.6	9.9	2.7	8.8	3.3	9.8	3.4	9.9	3.5		3.2
20	6.7	2.7	9.3	2.8	8.6	3.5	9.9	3.2	9.8	3.7		3.1
21	7.3	2.9	8.9	3.1	8.5	3.8	11.0	3.5	9.8	3.7	8.5	3.1
22	6.9	2.6	10.2	3.0	9.6	3.7	10.0	3.3	11.0	3.6	10.0	3.0
23	6.7	2.6	9.7	3.0	8.9	3.5	10.1	3.1	8.8	3.8	10.4	3.3
24	7.8	2.8	10.2	3.0		3.4	10.1	2.8	10.2	3.5	10.4	3.1
25	7.5	2.9	9.9	2.7	9.3	3.3	9.9	2.9	10.2	3.5	10.6	3.0
26	7.4	2.8	10.2	2.7	9.1	3.3	9.7	2.6	10.4	3.5	10.5	2.7
27	6.9	2.5	9.9	2.3	9.5	3.0	10.1	3.0	10.5	3.2	10.3	2.9
28	6.5	2.4	9.8	2.4	8.9	3.2	9.8	3.0	10.2	3.5	10.7	2.9
29	7.0	2.6	9.9	2.8	9.1	3.2	9.6	2.1	9.9	3.4	10.0	3.1
30	6.7	2.4	9.6	3.1	8.7	3.5	9.0	3.0	9.9	3.3	9.6	3.1
31	5.6	2.3	9.3	2.7	8.3	3.4	8.7	3.1	9.2	3.4	9.2	3.2
32	5.5	2.1	9.2	2.8	8.2	3.6	9.1	2.8	8.2	3.7	10.0	2.7
33	5.3	2.2	9.0	2.4	8.2	3.2	8.7	2.7	10.9	3.4	9.2	2.7
34	4.9	2.2	8.7	2.5	8.1	3.4			9.8	3.4		
35	3.7						9.1				10.2	
36	3.3		9.7		8.9		8.9		9.9		10.4	
37	3.0		9.4		8.3				10.5			
38			9.4		9.6							
39					9.4							

[1] Milligrams per 100 ml.
[2] Animals placed on calcium-free diet.

TABLE 3

Effect of sham operation on serum calcium and magnesium in young sheep

Post surgery	X1		X2		X3		X4		X5	
	Calcium [1]	Magnesium [1]	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium
days										
0	10.0	2.4	10.5	2.2	10.5	2.6	10.5	2.7	10.0	2.8
1	8.7	2.2	9.6	2.6	10.4	2.9	10.0	2.9	9.9	2.8
2	8.7	2.6	10.6	2.7	9.9	2.8	9.6	2.9	9.6	2.7
3	9.9	2.6	10.0	2.7	10.3	2.8	10.1	3.1	9.4	2.8
4	10.0	2.5	10.0	2.6	10.3	2.9	10.3	2.9	9.4	2.9
5	10.9	2.3	10.2	2.7	10.1	2.8	10.1	3.1	9.6	2.7
6	11.2	3.5	10.5	2.9	10.3	3.1	10.0	3.1	9.7	2.6
7	11.2	2.8	10.6	3.1	9.7	3.0	10.0	3.0	10.8	3.1
8	11.4	2.9	12.5	3.3	10.4	2.9	10.9	3.0	10.3	3.0
9	11.0	3.0	11.2	2.9	7.2	2.9	10.7	3.0	11.5	3.3
10	11.5	3.2	9.9	2.9	8.6	2.8	11.8	3.4	10.7	3.2
11	11.0	3.3	9.8	3.0	9.6	2.6	11.1	3.4	10.8	3.0
12	8.9	2.8	11.1	3.0	10.3	2.8	11.3	3.2	10.9	3.1
13	8.7	2.9	10.9	3.1	10.4	2.8	10.5	3.0	11.2	3.2
14 [2]	10.9	3.3	10.3	3.1	10.0	2.7	11.1	2.9	10.9	2.9
15	10.9	3.3	10.1	3.1	9.2	2.7	10.8	3.0	11.8	2.5
16	10.4	3.4	9.8	3.1	9.4	2.9	12.1	3.0	11.9	3.3
17	10.2	3.4	9.6	3.1	9.0	2.8	12.0	3.8	11.2	3.2
18	10.2	3.2	8.8	3.1	9.6	3.0	11.6	3.2	12.0	3.3
19	9.8	3.2	9.9	3.3	10.4	3.0	11.2	3.2	11.6	3.6
20	10.6	3.1	9.8	3.4	10.2	2.8	11.7	3.2	11.7	2.9
21	11.0	3.1	9.3	3.0	10.2	2.9	10.9	3.0	11.2	2.8
22	10.9	3.2	10.6	3.5	10.2	2.8	10.8	3.1	12.4	2.7
23	11.5	3.1	10.7	3.3	10.8	3.1	11.7	3.2		
24	11.0	3.1	10.1	2.7	10.2	3.0				
25	11.7	3.3	10.9	3.0	10.4	3.1				
26	10.7	3.1	9.6	2.9	9.8	3.1				
27	11.6	2.8	9.9	3.4	10.4	2.8				
28	10.8	3.0	9.6	2.9	11.0	3.0				
29	11.9	2.6	10.6	3.0	10.6	2.9				
30	11.8	2.8	11.4	3.1	10.6	3.1				

[1] Milligrams per 100 ml.

[2] Animals placed on calcium-free diet.

100 ml to 7.8, but subsequently dropped until the animal died 43 days postoperation. Tetany was not observed. One superior parathyroid was left in sheep A3 and complete removal of one thyroid lobe in sheep A2 was not confirmed. Sheep A3 responded with a decrease in serum calcium only after being placed on the calcium-free diet, when there was a steady fall in serum calcium for 1 week. The serum calcium in sheep A2 and A3 remained lower than the sham-operated sheep after feeding the calcium-free diet.

In the sham-operated adult sheep serum calcium values were affected only when they were placed on the calcium-free diet (table 3). Differences in serum calcium levels between sham and thyroparathyroidectomized (TPX) adult sheep existed during the first postoperative week and after the feeding of the calcium-free diet.

Calcium balances on the adult sheep are shown in figure 1. Sheep A1 was in positive calcium balance during the first 2 weeks postoperation when the basal diet was being fed. During this same period, sheep A2 was in positive balance during week 1 but the balance became negative during week 2. Sheep A3, which retained one external parathyroid gland, was in negative calcium balance for both these weeks. The mean calcium balance for the sham-operated animals was negative for the same period. After feeding the calcium-free diet the calcium balance for all animals became negative. Sheep A1 was less negative than A2 < A3 < B1, B2 and B3. These data indicated that thyroparathyroidectomy decreased calcium mobilization from body stores and thereby decreased endogenous calcium excretion.

Complete thyroparathyroidectomy was performed in all five of the 6-month-old sheep; these were confirmed histologically. Control and postoperative serum calcium and magnesium values are shown in table 4. Serum calcium declined more rapidly in the young TPX sheep than in TPX adults. Feed consumption was excellent in the young sheep during the first 3 weeks postoperation. Sheep Y3 and Y4 died before being placed on the calcium-free diet. Sheep Y4 died during the night of day 13 after surgery, while sheep Y3 was observed

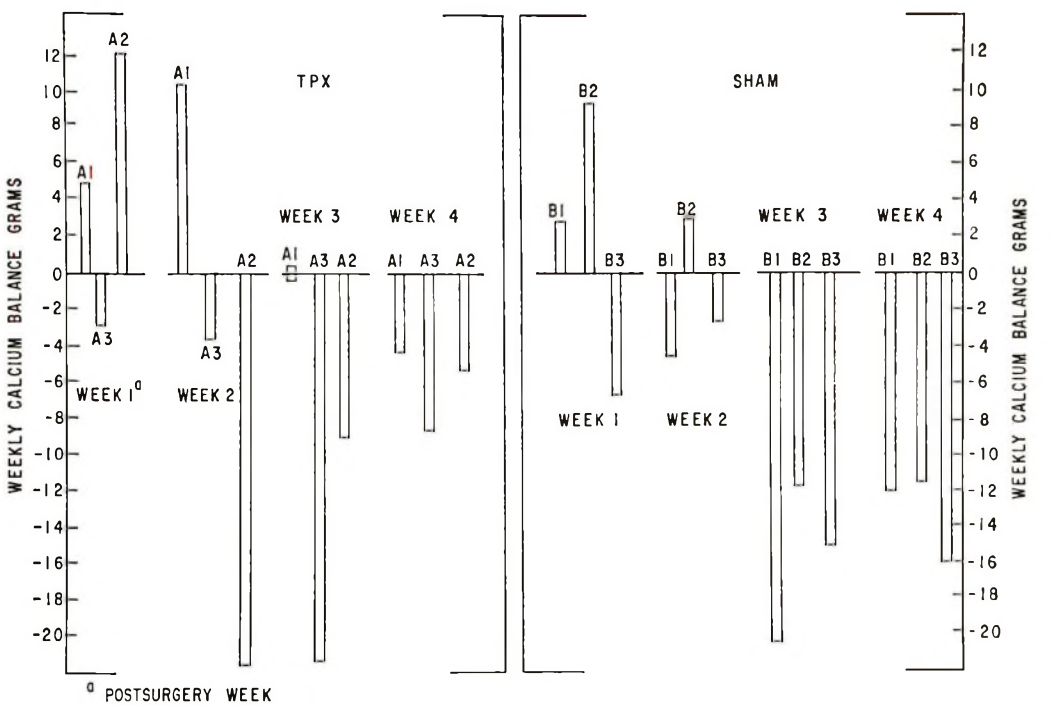

Fig. 1 Calcium balance for TPX and sham-operated adult sheep.

TABLE 4

Effect of thyroparathyroidectomy on serum calcium and magnesium in young sheep

Post surgery	Y1		Y2		Y3		Y4		Y5	
	Calcium [1]	Magnesium [1]	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium	Calcium	Magnesium
days										
0	11.0	2.5	10.6	3.3				2.4		
1	9.1	2.6	9.5	2.6	8.7	2.5	8.5	2.3	9.2	2.3
2	8.3	2.6	7.7	2.7	7.1	2.3	6.2	2.3	8.8	2.3
3	8.3	2.6	8.8	3.0	7.0	2.3	6.6	2.3	7.1	2.3
4	8.4	2.6	8.9	2.9	7.0	2.4	7.3	2.5	7.7	2.1
5	7.4	2.5	7.2	2.9	6.9	2.2	6.7	2.4	7.1	2.2
6	6.8	2.6	9.0	2.9	6.0	2.1	10.2	2.8	6.3	2.2
7	7.0	2.6	10.4	3.4	7.2	2.3	7.3	2.3	6.9	2.1
8	5.1	2.4	6.9	2.4	7.4	2.3	6.8	2.4	7.3	2.2
9	6.4	2.4	8.9	3.2	7.9	2.8	6.3	2.1	7.4	2.2
10	7.1	2.6	8.3	2.9	7.7	2.7	5.5	2.0	7.9	2.5
11	7.0	2.7	6.8	2.8	7.8	2.9	5.1	2.1	7.7	2.6
12	7.0	2.4	6.4	2.7	7.3	2.6	4.7	2.1	7.4	2.5
13	6.4	2.3	6.2	2.6	6.8	2.5	3.4	1.6	7.4	2.4
14 [2]	6.8	2.6	5.8	2.6	5.8	3.0			6.7	2.5
15	5.9	2.3	6.2	2.7	6.2	2.3			6.7	2.0
16	5.6	2.5	6.7	2.6					7.4	2.4
17	5.3	2.2	6.6	2.5					7.0	2.5
18	5.2	2.4	6.6	2.2					7.0	2.3
19	5.1	2.4	5.7	2.0					6.6	1.7
20	5.8	2.4	5.8	2.0					6.6	2.4
21	5.0	2.3	5.2	1.9					6.1	2.0
22	5.1	1.8	4.1						5.7	1.7
23	4.6	1.7							5.6	2.2
24	4.3	1.7							5.7	2.3
25	3.2	1.4							5.6	1.6
26									5.4	1.8
27									5.1	1.5
28									5.0	1.7
29									4.2	1.7
30									4.5	

[1] Milligrams per 100 ml.

[2] Animals placed on calcium-free diet.

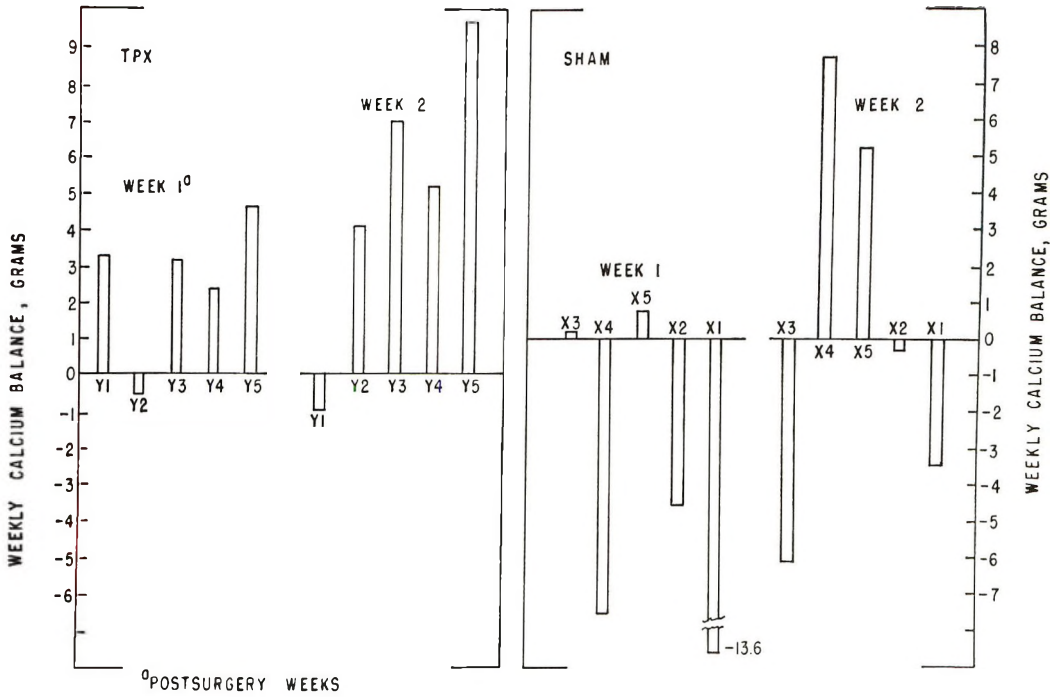

Fig. 2 Calcium balance for TPX and sham-operated young sheep.

going into tetany 15 days postoperation and died within 30 minutes of the onset of tetany. Sheep Y2 died on day 23 and Y1 on day 25, 9 and 11 days, respectively, after being placed on the calcium-free diet. Sheep Y5 died on day 34, 20 days after being placed on the calcium-free diet. This animal exhibited muscular weakness at day 21 and was unable to stand for several days. It recovered the ability to stand, but became recumbent again the day before death.

Figure 2 illustrates the calcium balance of the sham and TPX sheep during the first 2 weeks. Due to rapid mortality of the young TPX sheep, balance studies during the calcium-free diet phase could not be conducted. The TPX animals were in positive calcium balance following surgical removal of the glands, results which agree with those obtained with adult sheep A1 (trial 1).

Payne and Chamings (6) observed an initial fall in plasma magnesium of thyroparathyroidectomized goats followed by a return to normal. In these trials neither thyroparathyroidectomy nor feeding a calcium-free diet affected serum magnesium. Values decreased when feed intake decreased, the latter often ceased before death of the thyroparathyroidectomized sheep. Postmortem examination revealed no significant lesions in any of the animals.

DISCUSSION

Recent results (9, 10) indicate the importance of the parathyroid gland for calcium homeostasis in the ruminant animal. Thyroparathyroidectomy has been shown to be fatal in the young (4) but not the adult ruminant (5). The data presented in the present study indicate that age is an important criterion in assessing responses to thyroparathyroidectomy, and that the level of dietary calcium may be critical to the response mechanisms. The rate of decline in serum calcium is rapid in young TPX sheep and is often followed by fatal tetany, even when the animal is maintained on a normal diet. In contrast, TPX adult sheep are more tolerant to thyroparathyroidectomy, being capable of correcting hypocalcemia and maintaining serum calcium above sympto-

matic levels even after an extended time on a calcium-free diet. The higher calcium requirements for growing animals (11) may be a factor in this difference.

The positive calcium balance observed in young TPX sheep indicated that thyroparathyroidectomy does not appreciably affect absorption of calcium. Mayer et al. (9) have shown that administration of parathyroid extract in adult parathyroidectomized cows resulted in increased bone resorption and fecal endogenous calcium. Decline in serum calcium during positive calcium balance in young TPX sheep implies a reliance upon hormonal mobilization of storage calcium for maintenance of serum levels. This in turn indicates that the 0.4% calcium diet used in this experiment may not be adequate for sheep of this age.

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Fate of Threonine and Leucine in Rats Fed Threonine-deficient Diets

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ABSTRACT The incorporation of threonine-^{14}C and leucine-^{14}C into liver and muscle protein of young rats was determined after 3 hours and 2 weeks of feeding two different threonine-deficient diets. The rate of incorporation of two amino acids into the tissue protein was compared by calculating the relative specific activities; the ratio of specific radioactivity of the protein to those of the acid-soluble fraction in both liver and muscle. It was found that the incorporation of leucine-^{14}C into protein decreased in both liver and muscle of rats fed the threonine-deficient diets, whereas incorporation of threonine-^{14}C into protein was increased by feeding the same diets. Because a close parallel was found between body weight gain and relative specific activities of the tissue protein when leucine-^{14}C was injected, it was observed that tissue protein values in rats injected with leucine-^{14}C are a better index of the rate of protein synthesis in rats fed the threonine-deficient diets, than the rate of incorporation of threonine-^{14}C into tissue protein. The high value of the relative specific activities of the protein observed when the threonine-^{14}C was injected appeared to indicate the increased utilization of threonine in the animals fed the threonine-deficient diets. Metabolic adaptation by the rats fed the threonine-deficient diets has been discussed based on the experimental findings.

The metabolic effect caused by the amino acid imbalance has been studied by Yoshida et al. (1) and Hartman and King (2). These workers have demonstrated that incorporation of isotopically labeled limiting amino acids into tissue protein of rats was stimulated by the low protein diets, in which an amino acid imbalance was created by adding various amino acids except threonine or histidine. In an investigation on the amino acid imbalance, however, Kumta and Harper (3) reported that the body weight of rats decreased immediately after changing the balanced diet to the imbalanced diet, and recovered slowly to the normal level later. In an attempt to investigate the biochemical nature of such a diphasic change in the body weight, the rate of isotope incorporation of the limiting amino acid and other amino acids into tissue protein was determined using threonine-U-^{14}C and leucine-U-^{14}C, in rats fed two types of diets in which threonine was limiting.

EXPERIMENTAL

Feeding schedule of rats. Male rats of the Donryu strain (40 to 50 g) were used throughout the experiment. The animals were fed the basal diet (4) for 7 days (table 1). The rats were divided into five groups depending on the different treatments used. Group 1 served as the control, in which the basal diet was given continually for another 2 weeks. Groups 2 and 4 were used for the short-term feeding experiment. After a 24-hour fast group 2 was fed threonine-deficient diet 1 for 3 hours, and group 4 was fed threonine-deficient diet 2 for 3 hours. Groups 3 and 5 were fed threonine-deficient diets 1 and 2, respectively, for 2 weeks without prefasting. At the end of the feeding periods, rats were fasted for 24 hours; each respective diet then was given for another 3 hours. Then, either L-threonine-U-^{14}C (4.4×10^6 dpm) or L-leucine-U-^{14}C (8.86×10^5 dpm) was injected intraperitoneally.

One hour after injection of the radioactive amino acid, rats were decapitated; the liver and a part of the gastrocnemius muscle were removed immediately. Radioactivity of the protein and the acid-soluble fraction in the respective tissues was measured. The expired CO_2 was collected separately during the hour after injection of the radioactive amino acid, in a mixture of

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TABLE 1

Composition of basal diet, threonine-deficient diet 1, and threonine-deficient diet 2

	Basal diet	Threonine-deficient diet 1	Threonine-deficient diet 2
	g/kg of diet	*g/kg of diet*	*g/kg of diet*
Amino acid mixture lacking threonine [1]	48.55	48.55	48.55
Essential amino acid mixture lacking threonine [2]	—	—	52.13
Threonine	2.64	1.76	1.76
Vitamin mixture [3]	8.50	8.50	8.50
Choline chloride	1.50	1.50	1.50
Salt mixture [4]	40.00	40.00	40.00
Corn oil	50.00	50.00	50.00
$NaHCO_3$	1.91	1.91	5.39
Sucrose	846.90	847.78	792.07
Vitamin E	0.10	0.10	0.10
Vitamin A	6,000 IU	6,000 IU	6,000 IU
Vitamin D	600 IU	600 IU	600 IU

[1] The amino acid mixture contained: (in grams per kilogram diet) L-arginine, 1.58; L-histidine·HCl, 1.95; L-isoleucine, 2.75; L-leucine, 4.00; L-lysine·HCl, 4.15; L-methionine, 1.95; L-cystine, 1.15; L-phenylalanine, 3.48; L-tyrosine, 1.15; L-tryptophan, 0.94; L-valine, 3.48; L-alanine, 2.18; L-aspartic acid, 4.20; L-glutamic acid, 9.62; glycine, 1.59; L-proline, 2.18; and L-serine, 2.18 (4).

[2] Composition was the same as that in the above described amino acid mixture.

[3] The vitamin mixture contained: (percentage in the mixture) thiamine, 0.059; riboflavin, 0.059; niacin, 0.294; Ca pantothenate, 0.235; pyridoxine·HCl, 0.029; menadione, 0.006; biotin, 0.001; folic acid, 0.002; vitamin B_{12}, 0.0002; inositol, 1.176; ascorbic acid, 0.588; and lactose, 97.551.

[4] Harper, A. E. 1959 J. Nutr., 68: 405.

ethanolamine and ethylene-glycolmonomethylether (1:2, v/v), and the radioactivity was determined by the method of Jeffay and Alvarez (5). Radioactivity of all samples was counted in a liquid scintillation spectrometer.[1]

Measurements of radioactivity in acid-soluble fraction and protein. A portion of the liver and muscle was homogenized in a glass homogenizer using 9 volumes of ice-cold 5.6% trichloroacetic acid (TCA) to fractionate the acid-soluble fraction and protein. The total radioactivity in the acid-soluble fraction was determined after drying 0.3 ml of the supernatant fraction in a counting vial, followed by the addition of 0.3 ml hyamine 10X and 10 ml phosphor solution (4 g 2,5 diphenyloxazole (PPO) and 0.1 g 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) in 1000 ml toluene). The precipitable fraction of the TCA-extracted tissues was washed twice with 5% TCA, heated at 90° for 15 minutes in the same solution, and washed again. Then it was treated twice with an ethanol–ether mixture (3:1) at 55° for 30 minutes, and finally washed twice with warm ether. The resultant precipitate was dried and a 2-mg subsample was put in a counting vial, dissolved in 1 ml hyamine 10X (6). Ten milliliters phosphor solution (4 g PPO and 0.1 g POPOP in 1000 ml toluene) were added and the radioactivity measured. Separately, a portion of the dried protein fraction was hydrolyzed with 6 N HCl at 110° for 24 hours to analyze the amino acid content and for the autoradiography experiment described below. Specific radioactivity of the protein in each experiment was calculated as dpm/μmole of either threonine or leucine.

For the determination of the content of free amino acids in the tissue, another weighed portion of liver and muscle was homogenized with 1% picric acid by the method of Stein and Moore (7). The extract of each group was pooled and an aliquot was passed through a column of Dowex 2-X8. A part of the effluent was partially hydrolyzed with 2 N HCl (100°, 3 hours) (8), and the hydrolysate was dried and redissolved in 5.0 ml citrate·HCl buffer at pH 2.2. The amino acid content was then determined by an amino acid analyzer.[2] Another aliquot of the column effluent was dried without partial hydrolysis and dissolved in a small amount of the same buffer. It was then subjected to paper chromatography using solvent system *t*-butanol–H_2O–methylethyl ketone–diethylamine (80:80:40:8, v/v) for the threonine-

[1] Packard Instrument Company Inc., Downers Grove, Ill.

[2] Beckman Model 120B, Beckman Instruments, Inc., Fullerton, Calif.

^{14}C injected group, and *n*-butanol–acetic acid–H_2O (120:30:50, v/v) for the leucine-^{14}C injected group. After development, a 1-cm segment of the chromatogram was used for the radioactivity measurements using a liquid scintillation spectrometer (9). To compute the specific radioactivity of the respective amino acid in the acid-soluble fraction, the percentage of the radioactivity of each amino acid to the total of the acid-soluble fraction was calculated from the autoradiogram. Then the radioactivity was divided by the concentration of each free amino acid.

RESULTS

Growth and food intake. The growth-response curve of rats fed the threonine-deficient diets is shown in figure 1. The body weight gain of animals fed threonine-deficient diet 1 was greater compared with that of threonine-deficient diet 2. The body weight gain in these two cases, however, was much less compared with the group given the basal diet. Results of the food intake of these three diets during the experimental periods are presented in table 2. It appears that food intake of rats fed threonine-deficient diet 2 was much less compared with rats fed threonine-deficient diet 1.

Concentration of free threonine and leucine in the liver and muscle. Table 3 shows the concentration of free threonine and leucine in the liver and muscle of rats fed each diet. The content of free threonine in liver was greatly decreased by feeding rats both threonine-deficient diets compared with the basal diet. The content of free leucine, however, was not greatly affected by the threonine-deficient diets. Essentially a similar pattern was observed in the concentration of both threonine and leucine in muscle.

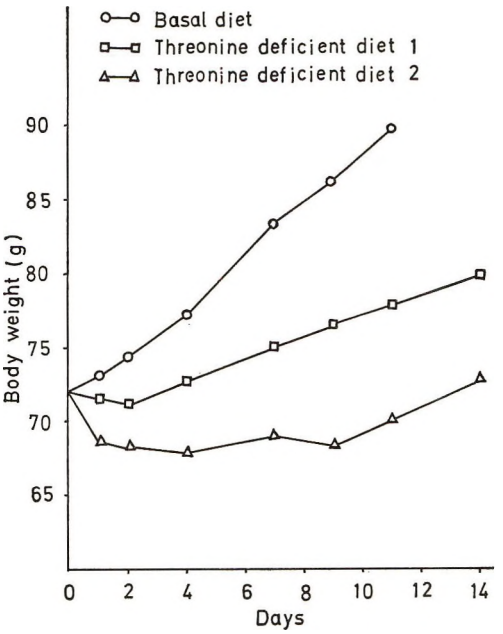

Fig. 1 Growth curve of rats fed the basal diet and two different threonine-deficient diets.

TABLE 2

Food intake of rats fed basal and threonine-deficient diets [1]

Diets	No. of rats	Growth periods, days			
		1–2	3–4	5–7	8–10
		Avg food intake/rat per day			
		g	*g*	*g*	*g*
Basal diet	4	7.3 ± 0.42 [2]	8.5 ± 0.69	8.4 ± 0.33	9.3 ± 0.49
Threonine-deficient diet 1	8	6.1 ± 0.34	7.1 ± 0.57	7.9 ± 0.27	8.3 ± 0.16
Threonine-deficient diet 2	8	4.1 ± 0.49	4.9 ± 0.36	6.1 ± 0.20	5.8 ± 0.31
		Avg food intake/body weight × 100/day			
Basal diet	4	10.2 ± 0.82	11.3 ± 0.95	10.6 ± 0.22	10.8 ± 0.44
Threonine-deficient diet 1	8	8.3 ± 0.42	9.5 ± 0.65	10.4 ± 0.32	10.6 ± 0.44
Threonine-deficient diet 2	8	5.9 ± 0.75	7.3 ± 0.59	9.0 ± 0.41	8.3 ± 0.24

[1] Food intake was measured every 2 or 3 days after feeding rats the basal diet.
[2] Mean ± SE.

TABLE 3

Concentration of free-threonine and leucine in liver and muscle of rats fed threonine-deficient diets

Group no.	Diets	Threonine		Leucine	
		Liver	Muscle	Liver	Muscle
		μmole/g tissue		*μmole/g tissue*	
1	Basal diet	0.532 [1]	0.760	0.130	0.060
	Threonine-deficient diet 1				
2	3-hour feeding	0.440	0.748	0.126	0.065
3	2-week feeding	0.124	0.300	0.130	0.039
	Threonine-deficient diet 2				
4	3-hour feeding	0.158	0.326	0.156	0.089
5	2-week feeding	0.200	0.348	0.137	0.068

[1] Each individual experiment was carried out using pooled liver or muscle of five rats.

TABLE 4

Percentage recovery of radioactivities in expired CO_2 after injection of threonine-U-^{14}C and leucine-U-^{14}C

Group no.		Recovery of radioactivities in expired CO_2	
		Threonine-U-^{14}C [1]	Leucine-U-^{14}C [2]
		%	%
1	Basal diet	5.24 ± 0.039 [3]	3.84 ± 0.393
	Threonine-deficient diet 1		
2	3-hour feeding	5.49 ± 0.225	4.52 ± 0.484
3	2-week feeding	5.50 ± 0.227	5.30 ± 0.376
	Threonine-deficient diet 2		
4	3-hour feeding	5.05 ± 0.127	10.72 ± 1.342
5	2-week feeding	4.78 ± 0.183	11.74 ± 1.810

[1] Threonine-U-^{14}C (4.40×10^6 dpm/rat) was injected intraperitoneally.
[2] Leucine-U-^{14}C (8.86×10^6 dpm/rat) was injected intraperitoneally.
[3] Mean ± SE.

Measurement of radioactivity in expired CO_2. Table 4 shows radioactivity measurements of the expired CO_2 1 hour after injection of either threonine-U-^{14}C or leucine-U-^{14}C to rats. Regardless of the different feeding schedules and feeding periods, not much variance was observed in the radioactivity of the expired CO_2 in the case of threonine-^{14}C. Only a slight decrease was encountered in group 5 fed threonine-deficient diet 2. Results, however, indicate the increased degradation of leucine-^{14}C to the expired CO_2 as shown in groups 4 and 5.

Incorporation of amino acids into acid-soluble fraction and protein. The liver protein isolated from rats, to which either threonine-^{14}C or leucine-^{14}C was injected, was hydrolyzed following the method described in the Experimental section. Results of the autoradiogram are shown in figure 2. In each case, the only detectable radioactive compounds were threonine and leucine. In liver, the content of threonine and leucine per milligram protein was 0.392 and 0.669 μmole; in muscle 0.314 and 0.555 μmole, respectively. Autoradiograms of the acid-soluble fraction isolated from liver and muscle of rats, to which either threonine-^{14}C or leucine-^{14}C was injected, are shown in figure 3. One of the two radioactive compounds in the threonine-^{14}C-injected group coincides well with threonine. In the case of the muscle fraction only discernible radioactive compounds were identified as threonine. When leucine-^{14}C was injected, however, five ra-

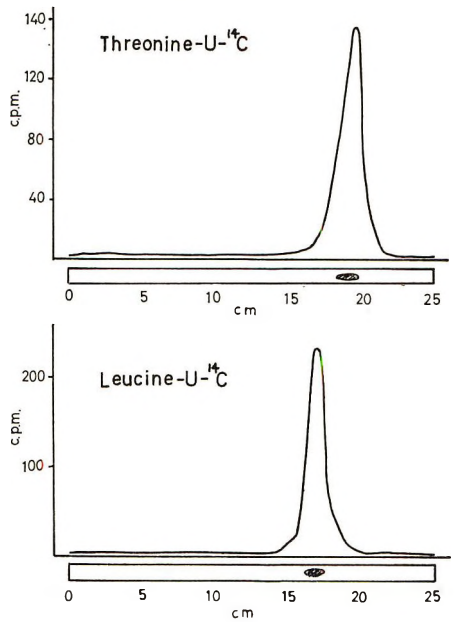

Fig. 2 Paper autoradiogram of protein hydrolysate of liver. Experimental details are explained in text.

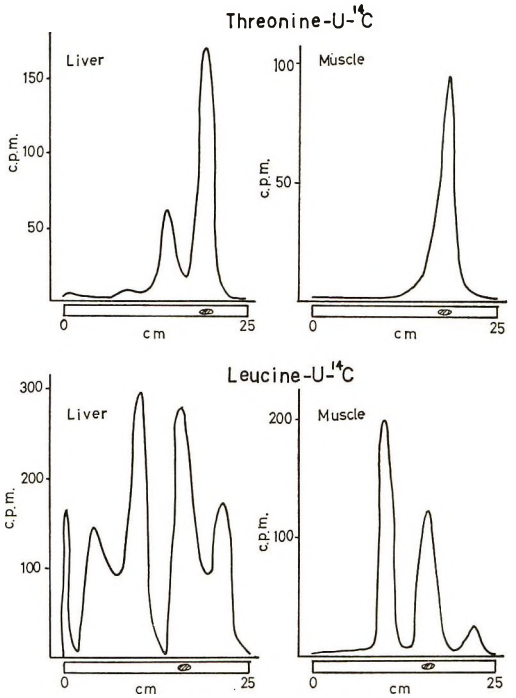

Fig. 3 Paper autoradiogram of acid-soluble fraction of liver and muscle. Experimental details, essentially similar to those shown in figure 2, are explained in text.

dioactive peaks were detected in the liver extract and three peaks in the muscle extract. In each case one of them was found to be leucine. The percentage of the radioactivity of each amino acid to total radioactivity in the acid-soluble fraction is presented in table 5.

Because the magnitude of the radioactivity incorporated into protein from amino acid-^{14}C is considered to be greatly influenced in vivo by the pool size of free amino acids (acid-soluble fraction), the relative specific activity of the protein (specific radioactivity of protein/specific radioactivity of acid-soluble fraction) was calculated from the data (table 6, columns

TABLE 5

Proportion of radioactive threonine and leucine in the acid-soluble fractions of liver and muscle

Group no.	Diets	Threonine		Leucine	
		Liver	Muscle	Liver	Muscle
		%	%	%	%
1	Basal diet	74 [1]	100	28	32
	Threonine-deficient diet 1				
2	3-hour feeding	65	100	29	33
3	2-week feeding	35	100	31	34
	Threonine-deficient diet 2				
4	3-hour feeding	50	100	30	39
5	2-week feeding	50	100	30	39

[1] The percentage was calculated from the autoradiograms of the acid-soluble fraction as exemplified in figure 3.

TABLE 6

Relative specific activities of liver and muscle protein of rats fed threonine-deficient diets

Diets	No. of rats	Liver			Muscle		
		Specific activity of protein (A) [1]	Specific activity of acid-soluble fraction (B) [2]	Relative specific activity (A)/(B) [3]	Specific activity of protein (A) [1]	Specific activity of acid-soluble fraction (B) [2]	Relative specific activity (A)/(B) [3]
		Threonine-U-^{14}C-injected groups (exp. 1)					
		dpm/μmole threonine	*dpm/μmole threonine* $\times 10^4$	*dpm/μmole threonine* $\times 10^{-2}$	*dpm/μmole threonine*	*dpm/μmole threonine* $\times 10^4$	*dpm/μmole threonine* $\times 10^{-3}$
Basal diet	4	1091 ± 27 [4]	5.67 ± 0.69	2.14 ± 0.39	141 ± 4.4	5.67 ± 0.66	2.89 ± 0.45
Threonine-deficient diet 1							
3-hour feeding	5	1280 [5]	4.48	2.86	110	3.46	3.18
2-week feeding	8	1055 ± 37	3.94 ± 0.10	2.82 ± 0.18	143 ± 16.5 [6]	2.17 ± 0.40	7.31 ± 1.77
Threonine-deficient diet 2							
3-hour feeding	5	1250 [5]	5.85	2.14	173	4.75	3.65
2-week feeding	8	1205 ± 65	3.79 ± 0.13	3.21 ± 0.22	136 ± 22.4 [6]	2.01 ± 0.45	8.11 ± 2.12
		Leucine-U-^{14}C-injected groups (exp. 2)					
		dpm/μmole leucine	*dpm/μmole leucine* $\times 10^4$	*dpm/μmole leucine* $\times 10^{-2}$	*dpm/μmole leucine*	*dpm/μmole leucine* $\times 10^4$	*dpm/μmole leucine* $\times 10^{-3}$
Basal diet	5	1774 ± 146	3.54 ± 0.15	5.06 ± 0.33	201 ± 12.2	3.35 ± 0.12	6.04 ± 0.47
Threonine-deficient diet 1							
3-hour feeding	5	1997 ± 162	4.16 ± 0.30	4.92 ± 0.53	173 ± 9.2	3.43 ± 0.19	4.94 ± 0.33
2-week feeding	5	1749 ± 147	3.96 ± 0.14	4.41 ± 0.36	190 ± 6.6	5.46 ± 0.15	3.48 ± 0.06
Threonine-deficient diet 2							
3-hour feeding	5	1502 ± 73	4.92 ± 0.29	3.07 ± 0.18	168 ± 17.6	3.85 ± 0.34	4.47 ± 0.62
2-week feeding	5	1818 ± 98	4.96 ± 0.27	3.71 ± 0.28	154 ± 6.9	5.78 ± 0.48	2.77 ± 0.33

[1] Specific activity of protein = $\frac{\text{dpm/mg protein}}{\mu\text{moles of threonine or leucine/mg protein}}$

[2] Specific activity of acid-soluble fraction = $\frac{\text{dpm/g tissue}}{\mu\text{moles of threonine or leucine/g tissue}} \times \frac{\text{percentage shown in table 5}}{100}$

[3] Relative specific activity = $\frac{\text{Specific activity of protein}}{\text{Specific activity of acid-soluble fraction}}$

[4] Mean ± SE.

[5] Experiment was carried out with pooled liver or muscle of five rats.

[6] Experiment was carried out with three rats.

3 and 6). It can be seen that protein values were increased, both in liver and muscle, by threonine-^{14}C injection to rats fed threonine-deficient diets 1 and 2. This trend appeared significant in the case of muscle at the 2-week feeding level. These values, however, decreased both in liver and muscle of the leucine-^{14}C-injected group, and the trend was quite conspicuous in muscle protein.

DISCUSSION

To review our observations concerning the relative ratio of the specific radioactivity of protein to those of acid-soluble fraction in rats fed the threonine-deficient diets (cf. table 6): In the case of threonine-^{14}C injection, the ratio of the group fed the threonine-deficient diet appeared to be higher compared with that of the group fed the basal diet. In the leucine-^{14}C-injected animals, however, the reciprocal relation was observed. These results may indicate that in rats fed the threonine-deficient diets, the fate of threonine is not the same as that of leucine in the animal body. There appears to be a good correlation, however, between body-weight gain of rats fed two different threonine-deficient diets and the relative specific activity of protein in the leucine-^{14}C-injected rats. Therefore, the relative specific activity obtained by the leucine-^{14}C-injected rats appears to be a more reliable index of protein synthesis in rats fed the threonine-deficient diets. Sidransky and co-workers (10, 11) conducted an analogous experiment using rats force-fed a threonine-devoid diet, to which various radioactive amino acids, e.g., leucine, isoleucine and valine, were injected. They observed in rats fed the threonine-devoid diets that the rate of protein synthesis decreased in the skeletal muscle, although the rate appeared to increase in the liver (10–13). Results of our present investigation, however, show that changes in the rate of protein synthesis, due to feeding threonine-deficient diets, exhibit a similar pattern in both liver and muscle, although the effect was more marked in the latter tissue. The difference between our results and those of Sidransky et al. may be due to different experimental conditions.

Both Harper et al. (14–16) and Ellison and King (17) have reported that the initial decrease in body weight in rats fed an amino acid-imbalanced diet might be caused by decreased food intake. They thus presented a hypothetical view that the very low content of the limiting amino acids in plasma may regulate the food intake of rats. Essentially a similar result was obtained in the present study. As shown in tables 2 and 3, parallel with the decrease in average food intake of rats fed threonine-deficient diet 2, threonine concentration was decreased in both liver and muscle. From the growth-response curve of rats fed the three diets (fig. 1), the rate of protein synthesis appears to be impaired more markedly by the 3-hour feeding of threonine-deficient diet 2 than the 2-week feeding. Actual results obtained, however, did not agree with this (table 6). Consequently, the initial depression of growth may be attributed to the decreased food intake of rats. The greater relative specific activity of protein obtained from the threonine-^{14}C-injected rats, to which the threonine-deficient diets were given, as compared with the group fed the basal diet, suggests two alternatives: (a) an enhanced protein synthesis, or (b) a greater turnover and reutilization rate of the threonine molecule. The former possibility is considered quite unlikely, because the data are not compatible with those obtained by the leucine-^{14}C experiment. Assuming the mechanism based on the second possibility (b) is operative, the respiratory breakdown of threonine to CO_2 would be expected to be lower compared with the one of other amino acids. The results shown in table 4 support this; the production of CO_2 from leucine-^{14}C was greater compared with that of threonine-^{14}C. Wilson et al. (18) and Yoshida et al. (1) reported that the metabolic breakdown of the limiting amino acids in rats fed the amino acid-imbalanced diets decreased. On the other hand, Yoshida et al. (1) and Hartman and King (2) observed the more efficient incorporation of the limiting amino acid into tissue protein of rats fed an amino acid-imbalanced diet compared with those fed the basal diet. Kean (19) reported the enhancement of activity of the methionine-activating enzyme in rats fed a methionine-

deficient diet. All these results appear to favor our findings, supporting the concept of the possible reutilization of threonine in rats fed the threonine-deficient diets. It should be noted, however, that despite the greater utilization of threonine the rate of protein synthesis in rats fed the threonine-deficient diets is still lower compared with that of the group fed the basal diet.

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Influence of Caffeine-containing Beverages on the Growth, Food Utilization and Plasma Lipids of the Rat [1]

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ABSTRACT It has been suggested that coffee-drinking may be a factor in the causation of cardiovascular disease, and tea drinking a factor in its prevention. In the present study, freeze-dried tea, decaffeinated coffee, coffee and pure caffeine were fed to rats, for 54 days, in a starch-based diet devoid of recognized atherogenic agents. None of the dietary supplements affected growth rate, the efficiency of food conversion or the weights of the organs. Changes produced in the plasma lipid values were proportional to the caffeine content of the diets. With increasing intake of caffeine, plasma cholesterol and phospholipid concentrations rose, while the triglyceride concentration fell. Feeding sucrose affected the plasma lipids in the opposite direction; triglycerides were increased whereas cholesterol and phospholipids were unaltered. This difference in response is attributed to a difference in the rate of lipid synthesis in the liver. The activity of pyruvate kinase, which was used as an index of lipogenesis, was unaffected by coffee, but was doubled by the substitution of sucrose for starch in the diet. Results are discussed briefly in relation to current views on the role of plasma lipids in atherogenesis.

Considerable interest has been shown in the possible role of caffeine-containing beverages in the etiology of cardiovascular disease. In a longitudinal study of some 2000 subjects, Paul et al. (1) found a positive relationship between coffee consumption and the development of coronary disease. Yudkin and Roddy (2) also reported that patients with myocardial infarction drank more tea and coffee than did an age-matched control group, but statistical analyses revealed that the association of the disease with the consumption of tea and coffee was due solely to the sugar taken with the beverages (3).

In the few experiments conducted on laboratory animals, diets containing cholesterol have been used. In the rabbit, both tea and coffee were found to reduce serum cholesterol levels and the severity of the cholesterol-induced atheromatosis (4, 5); in the rat, coffee ingestion promoted a rise in serum cholesterol, phospholipid and triglyceride concentrations, and tea a significant fall in these lipid fractions (6).

In the experiments reported here, the effect of caffeine-containing beverages on the plasma lipids of the rat was studied with a starch-based diet devoid of cholesterol. At the same time, a comparison was made between the effects on lipid metabolism of adding coffee to this diet, and replacing the starch with sucrose.

EXPERIMENTAL

The investigation was conducted in two parts. In the first part (exp. 1), the influence of dietary caffeine on growth and blood plasma lipids of the rat was determined. In the second part, a comparison was made between the effects of coffee and sucrose on plasma lipid patterns (exp. 2) and on the activity of hepatic pyruvate kinase (exp. 3), an enzyme believed to have a role in the control of lipid synthesis (7).

Experiment 1. Male rats of the Sprague-Dawley strain, from nine litters (each litter containing six or more males), were fed stock diet 41B [2] (8) until they attained an individual body weight approaching 120 g. The five pups in each litter, which during this time had remained closest in body weight, were assigned to five different dietary treatments, and transferred from stock diet to their experimental diets when they had reached 120 g.

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[1] Supported in part by a grant from General Foods.
[2] Haygates & Company, Bugbrooke Mills, Northampton, England.

Within any litter, all five animals began consuming the experimental diets within a few days of each other.

One rat from each litter received the control diet; [3] the remaining four rats in each litter were fed the control diet supplemented with coffee,[4] decaffeinated coffee,[5] tea or pure caffeine. The coffees were prepared by a standard method, then freeze-dried.[6] One cup (200 ml) of beverage contained 2.4 g coffee solids. The freeze-dried tea was a commercial product, one cup of which contained 1.0 g solids. The dried coffees and tea were added to the control diet at a concentration of 2.3 and 1.0 g/100 g diet, respectively. The fifth diet was supplemented with 140 mg pure caffeine per 100 g diet, an amount equal to the caffeine content of the supplement of dried coffee.

The amounts of the supplements consumed by the rats corresponded to a daily consumption of 12 cups of beverage by a 70-kg man. The calculation makes use of metabolic body weights (kilogram $^{0.75}$) and is based on an intake of 16 g of diet/day by a 200-g rat at the midpoint of the experiment.

The rats were housed under thermostatic conditions (22 ± 1°) in individual screen-bottom wire-mesh cages. They were weighed after 4 days, and thereafter at weekly intervals. Food consumption was measured on the days on which the animals were weighed.

After 54 days, the rats were fasted overnight then killed under ether anesthesia. Ten milliliters of blood were collected from the heart in a heparinized tube; the liver, kidneys, spleen, heart and adrenals were excised and weighed.

The total cholesterol concentration of the plasma was measured by the method of Zlatkis et al. (10) as modified by McIntyre and Ralston (11). Plasma phospholipids were determined by the method of Zilversmit and Davis (12) and triglycerides by the micromethod of Van Handel and Zilversmit (13).

The Wilcoxon matched-pairs signed-ranks test (14) was used to establish the significance of differences between the lipid values resulting from the various dietary treatments.

Experiment 2. The strain of rat, and the methods of selection and management used in experiments 2 and 3 were as described above.

Three rats from each of eight litters were transferred from stock to experimental diets when they weighed 140 g. Group 1 received the starch-based control diet, and group 2 the coffee-supplemented diet of experiment 1. In the diet of group 3, the starch was replaced quantitatively with sucrose.

After 54 days, the rats were killed following an overnight fast, and blood was drawn from the heart for lipid analyses.

Experiment 3. Three rats from each of six litters were fed, for 10 days only, diets of the same composition as had been used in experiment 2. The mean initial body weight of the rats in each group was approximately 80 g. Growth rate and food intakes were carefully measured. The animals were killed by a sharp blow on the head, and the livers were rapidly excised for estimation of pyruvate kinase activity.

Samples of liver were homogenized in 4 volumes of ice-cold 0.154 M KCl containing 0.66 mM EDTA. The homogenates were centrifuged at 15,000 rpm for 20 minutes. The activity of pyruvate kinase was measured in the supernatant fractions by the method of Valentine and Tanaka (15) modified to give a concentration of ADP in the reaction mixture of 2.4 mM. The reaction was followed at 340 μ in a spectrophotometer [7] at 25°.

[3] Diet: (in grams) casein, 24.0; arachis oil, 8.0; maize starch, 60.0; mineral mixture (9), 5.0; Solka Floc (cellulose powder), 3.0. The mineral mixture contained: (in grams) sodium chloride, 32.0; calcium hydrogen orthophosphate monohydrate, 130.0; tripotassium citrate monohydrate, 125.0; magnesium sulfate pentahydrate, 30.0; ferric citrate pentahydrate, 5.0; and 0.7 g of a mixture of trace elements composed of potassium iodide, 12.0; sodium fluoride, 10.0; manganous sulfate tetrahydrate, 2.0; cuprous iodide, 1.0; aluminum potassium sulfate, 1.0; and zinc sulfate heptahydrate, 1.0. To each kilogram of diet was added 2.25 g choline dihydrogen tartrate and 0.54 g of a mixture of vitamins of the B complex of the following composition: (in grams) inositol, 13.2; nicotinic acid, 6.0; calcium pantothenate, 6.0; *p*-aminobenzoic acid, 4.5; thiamine·HCl, 0.3; riboflavin, 1.8; pyridoxine·HCl, 0.48; biotin, 0.012; and folic acid, 0.06. In addition, each rat received, weekly, an oral dose of 0.1 ml arachis oil containing 300 IU vitamin A, 20 IU vitamin D_2, 3 mg α-tocopheryl acetate and 0.1 mg menaphthone.

[4] Maxwell House.

[5] Sanka.

[6] The freeze-dried coffees and tea were kindly prepared by Affined Foods.

[7] Unicam S. P. 800B, Unicam Instruments, Ltd., England.

RESULTS

Experiment 1. All dietary supplements caused an initial reduction in food consumption and in growth, coffee and caffeine having the most marked effect (table 1). From days 5 to 53 no notable differences in food consumption or in growth rates were observed. Thus, when the animals were accustomed to their diets, the efficiency of food utilization was essentially the same for all groups.

The differences between the body weights of the groups fed the various supplemented diets and those of the controls persisted from day 4 to the end of the experiment (day 54). Consequently, final body weights were significantly reduced ($P = 0.025$ or less). In the case of the rats which received pure caffeine, the difference was slightly increased, so that, on day 54, their body weights were 10% lower than those of their controls (table 2).

None of the dietary supplements had any effect on the weights of the organs (table 2). The small differences that were observed could be accounted for by differences in final body weights, since the weights of the viscera are related to body weight.

Table 3 summarizes the results of analyses for lipids in the plasma of the rats.

Decaffeinated coffee had no effect on plasma lipids. As the caffeine content of the diet was increased, the values for plasma cholesterol and phospholipids rose, the largest amounts of caffeine (the coffee and caffeine groups) producing statistically significant changes. The values for plasma triglycerides for all the groups receiving dietary supplements, however, were lower than that for the controls, the differences being statistically significant for rats fed tea and caffeine.

Experiment 2. The addition of coffee to the starch-based diet affected the pattern of plasma lipids of the rat differently from the change brought about by replacing the dietary starch with sucrose (table 4, fig. 1).

In experiment 1, feeding coffee produced a significant rise in the plasma cholesterol and phospholipid fractions, but did not alter the concentration of triglycerides. Conversely, the plasma triglyceride concentration rose following the incorporation of

TABLE 1

Influence of dietary supplements of tea, decaffeinated coffee, coffee and caffeine on the rate of growth and on the efficiency of food conversion of young rats (nine rats per group)

Dietary supplement	Initial body wt	Wt after 4 days	Days 5 to 53		
			Wt gain	Mean daily food intake	Wt gain per 100 g diet
	g	*g*	*g*	*g*	*g*
None	121	147	181	19.1	19.7
Sanka	120	134	179	19.2	19.4
Tea	121	138	184	18.6	20.6
Coffee	121	122	188	18.1	21.6
Caffeine	121	123	174	17.6	20.6

TABLE 2

Mean values for organ weights of control rats, and rats fed tea, decaffeinated coffee, coffee or caffeine for 54 days (nine rats per group)

Dietary supplement	Final body wt	Liver	Kidneys	Heart	Spleen	Adrenals
	g	*g*	*g*	*g*	*g*	*mg*
None	321	8.29	2.49	1.26	0.67	58
Sanka	302	8.03	2.41	1.15	0.58	51
Tea	313	8.17	2.47	1.16	0.60	55
Coffee	298	8.12	2.47	1.09	0.58	52
Caffeine	289	8.08	2.22	1.12	0.54	53

TABLE 3

Median values for plasma lipids of control rats, and rats fed tea, decaffeinated coffee, coffee or caffeine for 54 days (nine rats per group)

Dietary supplement	Caffeine added per 100 g diet	Total cholesterol	Phospholipids	Triglycerides
	mg/ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
None	Control	85 (75–105) [1]	137 (124–149)	38 (23–65)
Sanka	4.8	85 (55–115)	143 (102–206)	34 (18–44)
Tea	77.9	95 (70–120)	159 (121–174)	29 (17–52)
Coffee	136.2	105 (80–130)	171 (126–191)	30 (19–42)
Caffeine	140.0	105 (90–140)	174 (131–190)	27 (20–41)

[1] Figures in parentheses give range of values.

Statistical analyses (P values)

Control versus Sanka	ns [2]	ns	ns
Control versus tea	ns	0.025	0.01
Control versus coffee	0.025	0.01	ns
Control versus caffeine	< 0.005	< 0.005	0.01
Coffee versus Sanka	0.005	0.05	ns

[2] ns = not significant.

TABLE 4

Median values for plasma lipids of rats fed starch, starch with coffee, or sucrose for 54 days (eight rats per group)

Diet	Total cholesterol	Phospholipids	Triglycerides
	mg/100 ml	*mg/100 ml*	*mg/100 ml*
Starch	59 (50–70) [1]	124 (100–139)	37 (28–39)
Starch/coffee	90 (65–122)	148 (121–183)	35 (29–45)
Sucrose	61 (50–72)	124 (105–135)	53 (42–78)

[1] Figures in parentheses give range of values.

Statistical analyses (P values)

Starch versus starch/coffee	0.005	0.005	ns
Starch versus sucrose	ns [2]	ns	0.005

[2] ns = not significant.

sucrose in the diet, but the cholesterol and phospholipid fractions were unaffected.

Experiment 3. It is believed that the blood plasma triglycerides may be synthesized exclusively in the liver (16). Because coffee and sucrose appeared to differ fundamentally in their influence on the plasma lipids, we sought an explanation for this difference in the metabolism of lipids in the liver. The activity of pyruvate kinase was measured in rats fed the same diets as were used in experiment 2; the results are shown in table 5.

Pyruvate kinase activity was not changed by the addition of coffee to the starch-containing diet. The replacement of starch with sucrose, however, doubled the activity of this enzyme ($P = 0.001$).

DISCUSSION

In most experiments designed to test the importance of diet in the development of cardiovascular disease, the amounts and proportions of the major serum lipid fractions have been the parameters most frequently measured.

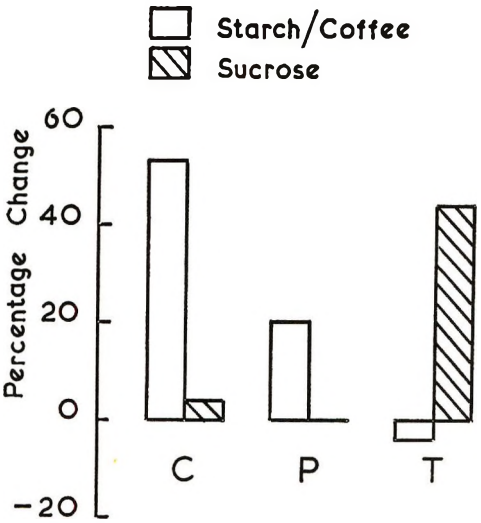

Fig. 1 Comparison of the effects on plasma cholesterol (C), phospholipids (P) and triglycerides (T) of supplementing a starch-based diet with coffee, and replacing starch with sucrose.

TABLE 5

Activity of pyruvate kinase in the livers of rats fed starch, starch with coffee, or sucrose (six rats per group)

Diet	Median	Range
Starch	7.25 [1]	6.45– 8.31
Starch/coffee	6.95	6.32–11.72
Sucrose	15.64	13.38–21.21

[1] Values of enzyme activity are given as micromoles of substrate converted per minute per gram of wet tissue.

Little and his colleagues (17, 18) in a retrospective study of the diet of male survivors of myocardial infarction, found a positive and statistically significant correlation between coffee consumption and serum lipid concentrations in their patients, although they failed to corroborate the original observation of Paul et al. (1) that coffee drinking was more common in such patients than in age-matched control subjects.

Changes in plasma lipid levels in the rat resulting from the feeding of tea and coffee have been described by Akinyanju and Yudkin (6). In a diet containing cholesterol, coffee had the effect of raising the concentrations of cholesterol, phospholipids and triglycerides, whereas tea caused a fall in all three fractions. As both these beverages contain caffeine, it was argued that caffeine could not be involved in the disturbances noted in the metabolism of the lipids.

Since the addition of cholesterol to a diet is known to raise the blood cholesterol level and suppress the synthesis of cholesterol in the liver,[8] the intervention of other dietary components in the metabolism of endogenous cholesterol might have been obscured. Furthermore, any substance in the diet that impaired the absorption of exogenous cholesterol would appear to exert a hypocholesterolemic effect.

In the present investigation, the influence of tea, decaffeinated coffee, coffee and caffeine on lipid metabolism was measured with a basic diet devoid of any of the agents commonly employed for the induction of experimental atherosclerosis. Under these conditions, the magnitude of the change in serum lipid concentrations was directly related to the caffeine content of the diet. The supplement of tea provided approximately half as much caffeine as the supplement of coffee, and caused a rise in plasma cholesterol that was half of that produced by the coffee. Decaffeinated coffee had no effect on plasma cholesterol; pure caffeine reproduced precisely the effects of coffee.

The rise in the concentration of phospholipids and the fall in the concentration of triglycerides were also in proportion to the intake of caffeine.

The changes in the plasma lipid concentrations brought about by tea and coffee in the absence of dietary cholesterol are therefore very different from those noted when cholesterol was fed (6). The apparent hypocholesterolemic effect shown by tea in the latter case may have been due to the impairment of cholesterol absorption by some component of tea, probably the tannins. The elevation of the plasma triglyceride level by coffee, but not by decaffeinated coffee, in the presence of exogenous cholesterol would appear to be an effect of caffeine which was not in evidence when cholesterol was eliminated from the diet.

There is a considerable body of evidence in support of the claim that sucrose may

[8] Gould, R. G., and C. B. Taylor 1950 Effect of dietary cholesterol on hepatic cholesterol synthesis. Federation Proc., 9: 179 (abstract).

be an important dietary factor in the development of coronary heart disease (19). Much of the sucrose is taken in association with tea and coffee, and the suggestion has been made (3) that the relationship found between the quantities of tea and coffee habitually consumed and the occurrence of heart disease may be due to the sugar consumed in these beverages.

Rats which have been fed sucrose show a rise in the plasma concentration of triglycerides, but no significant alteration in either total cholesterol or phospholipids (20). The change in the pattern of the plasma lipids following the administration of caffeine-containing beverages appeared, therefore, to be the converse of that resulting from sucrose feeding. This conclusion was confirmed in experiment 2.

It is probable that the disparity in the effects of dietary sucrose and caffeine on the plasma lipids results from differences in the rates of synthesis of the triglycerides in the liver. In experiment 3, the activity of hepatic pyruvate kinase was used as an index of lipogenesis. The addition of coffee to a starch-based diet had no effect on enzyme activity, whereas activity was doubled by substituting sucrose for starch in the diet.

The conclusions to be reached from these experiments with regard to the possible role of caffeine-containing beverages in the etiology of coronary heart disease must be based on the significance attached to alterations in the various plasma lipid fractions.

For many years, it has been recognized that patients with heart disease frequently have an elevated plasma cholesterol concentration (21). The increase in the plasma cholesterol of the rat resulting from the ingestion of tea and coffee might therefore imply that they are potentially harmful constituents of the diet. Recent reports have suggested, however, that a rise in the plasma triglyceride fraction is the more characteristic change encountered in atherosclerosis and coronary heart disease (22), in which case tea and coffee-drinking would be absolved.

The final judgment on these beverages must therefore be suspended until the mechanisms involved in the process of atherogenesis are clearly understood.

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Hypocholesterolemic Effect of Polysaccharides and Polysaccharide-rich Foodstuffs in Cholesterol-fed Rats [1]

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ABSTRACT The hypocholesterolemic effect of cellulose, sodium carboxymethylcellulose (CMC), pectin, alginic acid (free), agar-agar, gum arabic, konnyaku powder (prepared from the tubers of *Amorphophalus konjac*), konbu (*Laminaria japonica*), hijiki (*Hijikia fusiformis*) and aonori (*Enteromorpha prolifera*) was examined in rats fed hypercholesterolemic diets. The hypocholesterolemic effect was reconfirmed for pectin and a new demonstration of the plasma- and liver cholesterol-depressing activity was achieved for CMC and konnyaku powder. Absorption of cholesterol was significantly depressed in rats fed pectin and konnyaku powder, but rats fed CMC showed no alteration in cholesterol absorption as compared with the control group. The activity of konnyaku powder which is known to be hydrolyzed by intestinal microorganisms did not increase by the combined administration of antibiotics. From these facts, it appears that the mechanisms depressing plasma cholesterol differ appreciably in these three substances.

Many investigations have revealed that there is an intimate relationship between the plasma cholesterol level and atherosclerosis. The effects of dietary nutrients, and especially different types and levels of fat, protein and carbohydrate sources on the plasma cholesterol level, have been extensively studied.

Recently, Wells and Ershoff (1, 2), Fisher et al. (3), Leveille and Sauberlich (4) and Riccardi and Fahrenbach (5) reported on the hypocholesterolemic or antiatherosclerotic activity of pectin when added to a diet containing cholesterol. Fahrenbach et al. (6) also reported the plasma cholesterol-depressing activities of various mucilaginous polysaccharides in chickens fed cholesterol. Kaneda et al. (7–9) found that several seaweeds and mushrooms used as food in Japan had similar effects and studied the active principle(s) in the latter. The hypocholesterolemic activities of various synthetic derivatives of cellulose or dextran (anion exchangers) were also examined (10). The above findings suggest that other "effector(s)" with hypocholesterolemic activity rather than nutritional value may be present in normal foodstuffs which contain relatively larger amounts of some polysaccharides, but which are considered nutritionally poor by reason of their lower digestibility.

The present paper reports studies on the hypocholesterolemic activities of sodium carboxymethylcellulose (CMC),[2] cellulose,[3] pectin,[4] alginic acid,[5] agar-agar,[6] gum arabic,[7] konnyaku powder (*Amorphophalus konjac*),[8] konbu (*Laminaria japonica*),[9] hijiki (*Hijikia fusiformis*) [10] and aonori (*Enteromorpha prolifera*).[11] The activity of pectin was confirmed and the activities of sodium carboxymethylcellulose and konnyaku powder were discovered. "Konnyaku," a normal Japanese foodstuff derived from the tubers of *Amorphophalus konjac*, is made by swelling and dissolving in water, and then alkaline coagulation of konnyaku powder.

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[1] Part of this report was presented at the 22nd Annual Meeting of the Japanese Society of Food and Nutrition, May 19, 1968, Tokyo, Japan.

[2] Wako Pure Chemical Ind., Ltd., Osaka, Japan.

[3] 200–300 mesh powder, Toyo Roshi Kaisha, Ltd., Tokyo, Japan.

[4] Citrus pectin. Methoxyl content was not determined. Wako Pure Chemical Ind., Ltd., Osaka, Japan.

[5] This was free acid. Ishizu Pharmaceutical Company, Ltd., Osaka, Japan.

[6] Jelly intensity = 450–600 g/cm^2. Nakarai Chemicals, Ltd., Kyoto, Japan.

[7] Japan. Pharmacop.

[8] Isolated from tubers of *Amorphophalus konjac*, composed mainly of glucomannan which is known to give 1 mole glucose and 2 moles mannose on acid hydrolysis. Nitrogen content was 0.45%.

[9] *Laminaria japonica* (brown algae), a habitual Japanese foodstuff.

[10] *Hijikia fusiformis* (brown algae), a habitual Japanese foodstuff.

[11] Green algae, a habitual Japanese foodstuff.

EXPERIMENTAL

Male rats of the Wistar strain, weighing about 100 g, were used throughout. The average initial body weight of animals used in each experiment is given in the footnote of each table. There were five rats per group in all experiments except those shown in table 4. Animals were housed in individual cages with screen bottoms and fed a basal diet (20% casein) for 5 to 8 days. Room temperature was maintained at 23° ± 2°. Test diets were fed for the periods shown in each table; compositions of typical diets are shown in table 1. The type and dietary level of the fat source differed somewhat in different experiments, but other dietary constituents were essentially similar. The control diet (hypercholesterolemic diet) was prepared by supplementing the normal 20% casein basal diet with 1% cholesterol plus 0.25% bile salts. Test diets were prepared by the further addition of 5% test substances to the control diet, and a 5% reduction in carbohydrate sources. Fresh food was given ad libitum every morning. On the last morning of the test period, animals were anesthetized by intraperitoneal injection of 5% sodium pentobarbital solution.[12] Blood was taken by heart puncture into a heparinized syringe and centrifuged. Individual plasma cholesterol levels were measured by the method of Pearson et al. (11). After withdrawing the blood, livers were excised; they were blotted to remove excess blood and stored in a deep-freezer until they were analyzed. To minimize time deviations between the groups during these treatments, individual animals were taken from each group in turn and operations were carried out as quickly as possible. Feces were collected the last 3 days of the experimental period, dried at 60° in a hot-air current, and stored in a desiccator until they were analyzed.

Liver cholesterol was determined by the method of Pearson et al. (11) on an aliquot of the total lipid extracted with chloroform – methanol mixture (2:1,v/v) using the method of Folch et al. (12). A portion of the dried, ground feces was similarly extracted with chloroform–methanol mixture and Liebermann-Burchard reaction-positive sterols were determined by the procedure used for plasma and liver cholesterol.

Intestinal absorption of cholesterol was calculated in each group, making a correction for the cholesterol excreted by the normal group fed a cholesterol-free diet.

TABLE 1

Percentage composition of normal (basal), control and test diets

Constituents	Normal [1]	Control	Test
	%	%	%
Casein	20	20	20
Corn oil [2]	5	5	5
HPO [3]	(20)	(20)	(20)
Salts-B [4]	4	4	4
Vitamin mixture [5]	1	1	1
Choline chloride	0.15	0.15	0.15
α-Starch [6]	35	34.3	31.8
Sucrose	35	34.3	31.8
Cholesterol	—	1	1
Bile salts	—	0.25	0.25
Test substance	—	—	5

[1] This diet was also used in the preliminary feeding period.
[2] Mixed with vitamin A palmitate and vitamin D dispersed in water and α-tocopheryl acetate to provide 6000 IU, 600 IU and 100 mg/kg diet, respectively, prior to preparing the diet.
[3] HPO = hydrogenated palm oil, used only in experiment 1 as the dietary fat.
[4] This was identical with Harper's mixture (17).
[5] This was identical with Harper's mixture (17), but lactose was used as the bulking agent.
[6] This is a pregelatinized starch made from potato-β-starch.

RESULTS

First, the dietary conditions resulting in hypercholesterolemia were examined (table 2). Although data are not shown, the growth rates and food intakes of all groups were in the normal range. In the normal group receiving 5% corn oil as a dietary fat source, the average plasma cholesterol level was 108 mg/100 ml, and it did not increase when the diet was supplemented with 1% cholesterol alone (117 mg/100 ml). With the addition of 1% cholesterol with bile salts, however, there was a significant increase to 155 mg/100 ml ($P < 0.001$). This latter condition has been generally used for production of hypercholesterolemia and the value obtained here is in agreement with the values of other workers. When 20% hydrogenated palm oil was used as the fat source, however, the single addition of 1% cholesterol increased the plasma cholesterol level significantly (160 mg/100 ml); and with

[12] Nembutal.

TABLE 2

Preliminary experiment on dietary conditions elevating the plasma cholesterol level [1]

Dietary fat source	Addition	Cholesterol level	
		Plasma	Liver
		mg/100 ml	*mg/g tissue*
5% corn oil	None [2]	108 ± 4.7 [a,3]	4.1 ± 0.3 [a]
5% corn oil	1% cholesterol	117 ± 3.7 [a]	12.8 ± 1.0 [b]
5% corn oil	1% cholesterol + 0.25% bile salts	155 ± 6.5 [b]	46.5 ± 5.5 [c]
20% HPO	1% cholesterol	160 ± 9.2 [b]	8.6 ± 0.7 [d]
20% HPO	1% cholesterol + 0.25% bile salts	373 ± 21.0 [c]	31.2 ± 2.4 [e]

[1] The average initial body weight was 125 g (range: 100 to 140 g), and the feeding period, 2 weeks (five rats per group).
[2] This is identical with the normal diet listed in table 1.
[3] SE of the mean and figures with different alphabetical superscripts are significantly different ($P < 0.001$–0.05).

further addition of bile salts the level increased greatly, up to 373 mg/100 ml ($P < 0.001$). This latter dietary condition was given to the control group in experiment 1.

Experiment 1. After rats were fed the stock diet for 8 days, they were fed test diets for 2 weeks. The dietary fat was 20% hydrogenated palm oil. Table 3 shows that body weight gain and food intake in the test groups were rather less than those of normal or control groups, but no other abnormalities were observed. The rise in the plasma cholesterol level observed with the control diet (305 mg/100 ml) was depressed by supplementation of the diet with CMC (277 mg/100 ml), pectin (246 mg/100 ml) or hijiki (243 mg/100 ml), the differences being considerably lower but statistically not significant. Alginic acid had no effect on the plasma cholesterol level. Supplementation with cellulose and konbu (containing about 60% alginic acid on a dry weight basis), raised the plasma cholesterol levels appreciably above that of the control group. In the groups receiving CMC and pectin, less cholesterol was accumulated in the liver than in the control group. The liver cholesterol level appeared to parallel the plasma cholesterol level except in the group given hijiki. In animals receiving konbu or cellulose, the liver cholesterol contents like the plasma cholesterol contents were very high. The plasma cholesterol was unaffected by alginic acid, but the liver cholesterol content was more than the control value.

Experiment 2. In this experiment, the hypocholesterolemic activities of CMC, pectin and hijiki were reexamined and the activities of aonori, agar-agar and konnyaku powder were also tested. The dietary fat source was 5% corn oil. Previously, 2- to 10-week periods have been used in such experiments. To shorten the experimental period we examined the time required for elevation of the plasma cholesterol level by feeding a hypercholesterolemic diet, and for depression of the level by supplementation of active substances. Table 4 shows that on day 2 the plasma cholesterol had already increased to a maximum on a diet containing cholesterol plus bile salts, and was clearly depressed to the normal level when pectin was also given. Thus, the influences of these dietary supplements were reflected in the plasma cholesterol level shortly after ingestion of the diet. The results in table 4 are those of a preliminary experiment, and values are those of individual animals. Although this was a small-scale experiment, it shows that a change in the plasma cholesterol level appears soon after changing the diet. Probably the optimal test period varies with the mode of action of each test material and should be selected separately; however, a period of 8 days was used in experiment 2 for safety.

The data, summarized in the upper part of table 5, indicate that CMC and pectin, which lowered the plasma cholesterol in experiment 1, again showed remarkable depressing activity. The newly tested kon-

TABLE 3

Effects of various polysaccharides on food intake, body weight gain, and liver and plasma cholesterol levels in rats fed cholesterol [1]

Diet [2]	Cholesterol level		Avg daily food consumption	Avg body wt gain
	Plasma	Liver		
	mg/100 ml	*mg/g liver*	*g/rat/day*	*g/rat/2 weeks*
Normal	182±44.3 [3]	3.6±0.0	12.0±0.9	62.0±7.4
Control	305±68.9	26.8±2.9	11.1±0.6	50.0±6.9
CMC	277±10.4	20.0±2.8	10.7±0.7	49.8±5.7
Pectin	246±25.9	17.7±1.4 [4]	10.5±0.6	42.3±2.9
Alginic acid	301±26.9	32.3±2.9	10.4±0.0	43.0±2.9
Konbu	393±43.2	31.1±4.4	9.8±0.2	38.8±4.1
Hijiki	243±59.1	30.6±2.4	11.1±0.0	43.6±3.3
Cellulose	342±21.4	38.6±2.5	11.7±0.0	51.1±4.6

[1] The average initial body weight was 115 g (range: 101 to 128 g), and the feeding period, 2 weeks.
[2] The dietary fat source was 20% hydrogenated palm oil. The various supplements are listed in footnotes 2 to 11 of the text.
[3] SE of the mean.
[4] Significantly different from the control ($0.01 < P < 0.05$).

TABLE 4

Effect of length of test period on change in plasma cholesterol level [1]

Diet [2]	Plasma cholesterol level		
	Day 2	Day 4	Day 6
	mg/100 ml	*mg/100 ml*	*mg/100 ml*
Normal	124	114	95.8
Normal+1% cholesterol+0.25% bile salts	206	158	153
Normal	110	113	
Normal+1% cholesterol+0.25% bile salts +5% pectin	108	90.9	

[1] Average initial body weight, 100 g.
[2] Dietary fat source, 5% corn oil.

nyaku powder also gave a very low value. The differences between the plasma cholesterol values of the control rats and those fed these three substances were statistically significant ($P < 0.05$). The plasma cholesterol level in the group receiving hijiki was higher than that in the control group, unlike experiment 1. Aonori and agar-agar caused no depression. The liver cholesterol contents, like the plasma cholesterol levels, were also significantly lower in the groups receiving CMC, pectin and konnyaku powder ($P < 0.10$, $P < 0.01$ and $P < 0.05$, respectively). In groups receiving hijiki, aonori and agar-agar, in which the plasma cholesterol levels were high, the liver cholesterol was proportionately higher than that of the control group. In the case of pectin, one of the main factors depressing the plasma cholesterol level was considered to be interference with intestinal absorption of bile acids, and hence, with cholesterol (3). Indeed, in the present experiment (table 5), the corrected value for the percentage absorption in the group receiving pectin was very low, about one-half that of the control group ($P < 0.001$); konnyaku powder also significantly affected cholesterol absorption ($P < 0.01$). Even hijiki, aonori and agar-agar, which did not lower plasma cholesterol, produced less cholesterol absorption. On the other hand, rats fed a diet containing CMC, which showed a strong depressing activity, had a slightly higher percentage absorption of cholesterol than the control group.

Experiments 3 and 4. Repeat experiments on the effect of konnyaku powder did not give consistent results for plasma cholesterol, but the liver cholesterol content was about one-half that of the control group (lower part of table 5). As described in experiments 1 and 2, the lowered liver cholesterol content seemed to be related to the decrease in plasma cholesterol level. Because it appeared that dietary konnyaku

TABLE 5

Effect of various polysaccharides on food intake, body weight gain and plasma and liver cholesterol levels in rats receiving cholesterol [1]

Diet [2]	Cholesterol level		Cholesterol absorbed [3]	Absorbed	Avg daily food intake	Body wt gain for 8 or 9 days
	Plasma	Liver		Ingested		
	mg/100 ml	*mg/g liver*	*mg/rat/3 days*	*%*	*g/rat/day*	*g/rat*
			Exp. 2			
Normal	96.8 ± 0.4 [4,5]	3.2 ± 0.1 [6]	—	—	13.2 ± 0.6	38.3 ± 2.1
Control	156 ± 14.0	21.1 ± 2.0	288 ± 15.1	53.5 ± 1.0	13.5 ± 0.7	40.6 ± 4.7
CMC	110 ± 3.0 [7]	14.1 ± 2.6	220 ± 6.4	60.2 ± 6.3	12.1 ± 0.9	36.8 ± 3.6
Pectin	111 ± 9.2 [7]	11.1 ± 1.9 [5]	115 ± 20.8	28.0 ± 4.3 [6]	13.1 ± 0.3	37.0 ± 5.4
Hijiki	190 ± 24.0	23.2 ± 2.1	207 ± 21.5	41.2 ± 3.4 [5]	16.3 ± 0.5	51.2 ± 2.4
Aonori	187 ± 29.9	22.8 ± 2.3	173 ± 7.0	42.0 ± 2.3 [5]	14.1 ± 0.8	40.2 ± 3.8
Agar-agar	180 ± 21.9	25.5 ± 1.6	180 ± 15.5	38.3 ± 3.0 [5]	15.3 ± 0.6	40.8 ± 1.0
Konnyaku powder	107 ± 8.6 [7]	13.9 ± 1.8 [7]	139 ± 27.6	37.9 ± 6.3 [5]	13.1 ± 0.9	33.3 ± 4.5
			Exp. 3			
Normal	97.9 ± 2.9 [6]	2.6 ± 0.2 [6]	—	—	17.6 ± 1.1	44.8 ± 3.3
Control	137 ± 4.7	25.9 ± 3.4	—	—	16.1 ± 1.3	36.0 ± 6.6
Konnyaku powder	136 ± 10.6	10.4 ± 1.5 [5]	—	—	15.6 ± 1.2	34.4 ± 3.0

[1] The average initial body weight was 111 g (range: 100 to 123 g) and the experimental period was 8 days in experiment 2; the body weight was 140 g (range: 120 to 60 g) and the experimental period was 9 days in experiment 3.
[2] The dietary fat source was 5% corn oil.
[3] The absorption of cholesterol was corrected by subtracting the fecal excretion of Liebermann-Burchard reaction-positive materials in the normal group receiving no dietary cholesterol.
[4] SE of the mean.
[5] Significantly different from control group ($0.001 < P < 0.01$.
[6] Significantly different from control group ($P < 0.001$).
[7] Significantly different from control group ($0.01 < P < 0.05$).

powder exerted some effect, it was examined further (exp. 4). Moreover, because the variable results observed might be due to the degree of digestion of konnyaku powder by intestinal microorganisms, antibiotics were given as well.

The results in table 6, like those of experiment 2, indicate the distinct depression of plasma cholesterol by konnyaku powder to nearly normal levels. Addition of antibiotics did not increase the effect of konnyaku powder on plasma cholesterol, but gave an intermediary value between animals on the control and konnyaku powder diets. Gum arabic also exhibited moderate activity.

DISCUSSION

The effect of the following polysaccharides and Japanese foodstuffs containing polysaccharides on the plasma cholesterol level were examined in rats on a hypercholesterolemic diet: cellulose, sodium carboxymethylcellulose, pectin, gum arabic, konnyaku powder, alginic acid, agar-agar, hijiki, aonori and konbu. Among these substances, pectin, CMC and konnyaku powder exerted distinct and reproducible effects when added to the hypercholesterolemic diet at levels of 5%. In one experiment, konnyaku powder did not have a consistent effect on the plasma cholesterol level (table 5, lower part). The reason for this is uncertain but the konnyaku powder was purchased from a local dealer in small batches; those used in different experiments may have been different. When the effects of konnyaku powder on plasma cholesterol in all three experiments were combined and analyzed statistically there was a highly significant difference from the control values ($\phi = 28$, $P < 0.001$). Therefore, it may be concluded that konnyaku powder depresses plasma cholesterol. In experiment 1 hijiki appeared to depress plasma cholesterol but in subsequent trials consistent results were not obtained. Although gum arabic also exhibited appreciable activity in depressing cholesterol levels, results require confirmation. The same is true for alginic acid, agar-agar, konbu and aonori, which in single tests appeared to have no activity. This is especially so for aonori because Kaneda et al.

TABLE 6

Effect of konnyaku powder with and without antibiotics and of gum arabic on plasma and liver cholesterol levels in rats fed a hypercholesterolemic diet [1]

Diet [2]	Cholesterol level	
	Plasma	Liver
	mg/100 ml	*mg/g liver*
Normal	96.6 ± 5.7 [3]	2.8 ± 0.2
Control	212 ± 12.8	17.9 ± 1.0
Antibiotics [4]	231 ± 20.3	21.0 ± 1.2
Konnyaku powder	119 ± 7.0 [5,6]	13.8 ± 0.6 [7]
Konnyaku powder + antibiotics	170 ± 12.0 [7]	14.5 ± 1.1
Gum arabic	163 ± 10.0 [7]	17.9 ± 0.9

[1] The average initial body weight was 126 g (range: 118 to 137 g), and the feeding period was 5 days.
[2] The dietary fat source was 5% corn oil.
[3] SE of the mean.
[4] Sulfisomidin, 1.0%; dihydrostreptomycin sulfate, 0.1%; and oxytetracycline, 0.2% of the diet with a corresponding reduction in carbohydrate.
[5] Significantly different from control group ($P < 0.001$).
[6] Significantly different from normal group ($P < 0.05$).
[7] Significantly different from control group ($P < 0.05$).

(7) reported that aonori and other edible green seaweeds have considerable activity. This discrepancy may be due to different test conditions, such as differences in the feeding period, age and strain of test animals and the preparations tested. In the present experiments, calorie deficiency alone cannot account for the observed depression of the plasma and liver cholesterol levels, since there was little difference in food consumption in the three groups and in the control group (tables 3 and 5); on the contrary, the lowest food consumption was observed in rats receiving konbu, which had no beneficial effect. Moreover, all groups grew normally.

The effect on plasma cholesterol appeared much less with 20% hydrogenated palm oil than in later trials with 5% corn oil. The high dietary level of hydrogenated palm oil (which is abundant in saturated higher fatty acids) may itself cause an increase in plasma cholesterol even when cholesterol and bile salts are not added. In contrast, corn oil has no such effect (table 2). For example, the absolute amounts of decrease relative to the control in the group fed pectin were almost the same (tables 3 and 5): the absolute decrease in the group fed pectin with hydrogenated palm oil was 305–246 = 59 mg/100 ml whereas with corn oil it was 156–111 = 45 mg/100 ml.

Leveille and Sauberlich (4) found in experiments in vitro that pectin inhibited intestinal absorption of bile acid (taurocholic acid), and thus considered that it indirectly reduced the plasma and liver cholesterol levels by prevention of absorption of bile acid, although results of their experiment in vivo were not necessarily in agreement with this. The present data support the assumption that pectin acts as a hypocholesterolemic agent by impairing intestinal cholesterol absorption, since dietary pectin significantly lowered cholesterol absorption to one-half that in the control group (table 5).

Incorporation of konnyaku powder into the hypercholesterolemic diet markedly lowered the plasma cholesterol level. In this case, intestinal absorption of cholesterol was significantly reduced ($P < 0.01$) as compared with the control group (table 5). The hypocholesterolemic activity of konnyaku powder may be due to impairment of cholesterol absorption, but the detailed mechanism of its action is still obscure. Pectin generally is considered to be not digested and its effect is probably limited to an effect on the gastrointestinal tract, as conjectured by Leveille and Sauberlich (4). It was shown long ago, however, that in human subjects ingested konnyaku is almost completely digested, although the digestive juices do not break it down into assimilable forms. The hydrolysis of konnyaku mannan is achieved only by the action of intestinal microorganisms, as shown in man by Maeda (13), Nakamura (14) and Inoue (15), and the latter author isolated and identified *Aerobacter mannanolyticus* and other bacteria producing konnyaku mannan hydrolyzing enzyme(s) from human feces (16). Thus, the question arises whether konnyaku mannan has activity before or after hydrolysis. Since the simultaneous administration of antibiotics with konnyaku powder did not decrease the plasma cholesterol level more than konnyaku powder alone, the presence of intact konnyaku mannan molecules does not seem to be an absolute requirement in depression of plasma cholesterol. Thus, hydrolysis products of konnyaku mannan may play a role in depressing plasma cho-

lesterol either inside or outside the body, though it is also possible that intact mannan itself may act in the upper part of the intestinal tract prior to its hydrolysis, because its hydrolysis appears to be most rapid in the cecum.

Unlike pectin and konnyaku powder, when CMC was added to the hypercholesterolemic diet, there was about 60% cholesterol absorption which was somewhat higher than in the control group. The amount of cholesterol actually absorbed was about the same in the latter two groups, whereas the plasma cholesterol level was significantly lower with CMC than without CMC. It appears that the cholesterol-depressing mechanism of CMC may be considerably different from that of the other two active polysaccharides. At least, the most important effect of CMC would not appear to be its influence on cholesterol absorption. Therefore, it is probable that these three substances act in different and specific ways on cholesterol metabolism, including the impairment of intestinal absorption of cholesterol. Further studies are needed on this.

Among the "nonessential" or "nonnutritive" constituents of foodstuffs of higher animals, there may be many other substances which affect metabolic processes.

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Studies on the Cause of a Hemorrhagic Syndrome in Rats Fed a Water-soluble Chemically Defined Diet [1]

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ABSTRACT A hemorrhagic syndrome was observed in male CDF rats fed chemically defined liquid diets containing 2.1 mg/liter menadione. The syndrome occurred without resorting to surgery, coprophagy prevention, chemotherapeutic agents or antibiotics. It was observed in CDF rats only, although rats of CFE and Fischer strains were also tested. Ethyl cysteinate·HCl, a water-soluble form of cysteine was found to be the causative factor. The incidence and severity of the syndrome were related to its concentration in the diet. The underlying mechanism was found to be the interaction of the free thiol groups of ethyl cysteinate·HCl with menadione resulting in vitamin K deficiency. Substitution of equimolar amounts of diethyl cystinate·2HCl for ethyl cysteinate·HCl or menadiol sodium diphosphate for menadione, prevented the condition. Addition of 2-methyl, 3-cysteinyl, 1,4-naphthoquinone to the diets of susceptible and resistant rat strains, suggested that it is an inactive form of menadione rather than an antimetabolite. The severity of hemorrhagenicity was increased slightly by the addition of an oxygenated fat mix to the diet, and markedly by the simultaneous supplementation of vitamins C and E. When equivalent quantities of each vitamin were added alone they were ineffective. Exclusion of vitamin A from the diet did not influence the results.

Whenever nutritional hypoprothrombinemia has been produced in male rats in the absence of surgery, coprophagy prevention, chemotherapeutic agents or antibiotics, the diets used have either been very low or void in vitamin K-active compounds (1–3). Recently, without resorting to these conditions we observed a hemorrhagic syndrome in male, CDF Fischer rats fed a chemically defined liquid diet containing presumably an adequate concentration of menadione.[2] The syndrome was observed after rats had consumed the diet for 2 weeks and was manifested by internal and external bleeding and prolonged prothrombin times. Intramuscular injection of menadiol tetrasodium diphosphate prevented hemorrhage and restored prothrombin times to normal. Because menadione was present in the diet the therapeutic effect of the phosphate salt suggested destruction or interference, or both, with menadione utilization. Though many factors influence the vitamin K requirement of the rat, none of those known could account for this situation (5, 6). The experiments described here were designed to elucidate the factor(s) responsible for the vitamin K deficiency.

METHODS

Male CDF (Fischer derived) weanling rats [3] were used in all studies. Male CFE (Sprague-Dawley derived) rats [4] and male Fischer rats [5] were also used in two experiments. The duration of each experiment, and the age, strain and number of animals used are described in the tables of results. The rats were housed two per wire-bottomed cage in a temperature-controlled room (72 to 76°) and were allotted to insure, as closely as possible, uniform distribution of littermates within each dietary treatment. Diet and water were provided ad libitum. All liquid diets were fed via Richter tubes.[6] Residual diet was measured

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[2] Levenson, S. M., R. Shapiro, E. Seifter, N. A. Rosenthal, A. L. Nagler and E. F. Geever 1966 Vitamin K deficiency in rats ingesting a chemically defined diet containing menadione and no antibiotics or chemotherapeutic agents. Presented in part at the VIIth International Congress of Nutrition, Hamburg, Germany.

[3] Obtained from Charles River Breeding Laboratories, Wilmington, Mass.

[4] Carworth, New City, N. Y.

[5] A. R. Schmidt, Madison, Wis.

[6] Inverted glass drinking tubes graduated from 5 to 100 ml in 1-ml subdivisions. Designed by Dr. C. P. Richter, Johns Hopkins Hospital, Baltimore, Maryland.

and discarded daily and clean Richter tubes were then replenished with refrigerated diet. Fresh, glass-distilled water was provided on alternate days.

The water-soluble portion of the basal diet was identical to that reported by Greenstein et al. (6) except that it was supplemented with 2.4 g/liter NaCl and 1.75 g/liter $NaHCO_3$. The fat-soluble components of the diet were dissolved in the surfactant, polysorbate 80 [7] (2), and added to the diet to provide per liter: vitamin A acetate, 15,000 IU; *dl*-α-tocopheryl acetate, 25 IU; calciferol, 140 IU; menadione, 2.1 mg; ethyl linoleate, 2.0 g; and polysorbate 80, 3.0 g. The composition of the test amino acid mixtures (16 and 17) is shown in table 1. Diets containing one or the other of these mixtures are designated diet 16 or 17. Except for their amino acid composition and minor differences in glucose concentration the diets were identical. Glucose was varied to bring the final nutrient concentration of each diet to 50% weight by volume. All diets were prepared with glass-distilled water using techniques similar to those described by Greenstein et al. (7).

Guaiac tests (8) to detect internal hemorrhage were conducted daily on the feces under each cage. When the Guaiac tests were distinctly positive or if the animals showed signs of anemia, external bleeding or appeared moribund, they were anesthetized with chloroform and bled by heart puncture. The citrated blood samples were then centrifuged and plasma prothrombin times were immediately determined by the method of Quick (9).[8] Whenever prolonged prothrombin times were observed, Hicks-Pitney thromboplastin-generation time tests were performed to detect deficiencies in the stage 1 phase of the blood-clotting mechanism (10). The tables of results show the average and range of prothrombin times, the incidence of hemorrhagic death, and the number of abnormal animals in each group. Although prothrombin times do not follow a normal distribution pattern, conversion to reciprocal values (11) was not considered necessary for the purposes of this study, and would not basically influence the data. Prothrombin times were never run for more than 120 seconds. The average values reported for extremely depleted groups are, therefore, often lower than true values because they include animals with prothrombin times exceeding 120 seconds. Animals were considered abnormal if their prothrombin times and thromboplastin generation times were at least 66% longer than commonly observed for rats fed a stock diet. Death was considered hemorrhagic if autopsy showed evidence of either nasal, thoracic or subcutaneous bleeding.

TABLE 1

Composition of test amino acid mixtures

Amino acid [1]	Mixture 16	Mixture 17
	g/liter [2]	*g/liter* [2]
Arginine·HCl	4.7	8.9
Histidine·HCl·H_2O	2.85	3.38
Isoleucine	4.4	5.5
Leucine	7.0	7.3
Lysine·HCl	6.5	11.8
Methionine	3.15	5.39
Phenylalanine	3.15	7.63
Threonine	4.4	5.0
Tryptophan	1.4	1.5
Valine	4.9	5.5
Alanine	3.2	2.3
Asparagine	—	3.95
Aspartate, monosodium	7.94	2.7
Cysteine ethyl ester·HCl	0.55	2.43
Glutamate, monosodium	25.6	27.95
Glycine	2.05	13.94
Proline	12.7	2.3
Serine	6.6	2.3
Tyrosine ethyl ester·HCl	8.4	3.14

[1] All amino acids except glycine were of L configuration.

[2] Quantities provided in 1 liter of a 50% weight by volume complete diet solution.

RESULTS

The results of experiment 1 (table 2) corroborated our earlier observation that male, CDF rats ingesting diet 17 suffered from nutritional hypoprothrombinemia. Prothrombin times of these animals were prolonged and three hemorrhagic deaths occurred. Although the average and range of prothrombin times for rats fed diet 16 were greater than usually observed for animals ingesting this diet or a stock diet, only three animals were abnormal.

Our earlier studies indicated that diet 17 produced a vitamin K deficiency in CDF rats.[9] Because strain differences in sus-

[7] Tween 80, produced by Atlas Chemical Industries, Inc., Wilmington, Del.

[8] All assays were performed with a BBL automated Fibrometer System, Baltimore Biological Laboratory, Baltimore, Md.

[9] See footnote 2.

TABLE 2

Rat strain specificity in susceptibility to hypoprothrombinemia produced by a chemically defined liquid diet

Rat strain	Test diet	Prothrombin time		Abnormal rats	Hemorrhagic deaths
		Avg	Range		
		seconds	*seconds*		
		Exp. 1 [1]			
CDF [2]	16	19.4	10.8–61.8	3/10	1
	17	> 69.2	18.1– > 120	10/10	3
		Exp. 2 [3]			
CFE [4]	16	10.9	9.8–13.3	0/18	0
	17	10.3	9.8–11.8	0/18	0
	stock [5]	10.1	9.8–12.9	0/18	0
ARS [6]	16	10.7	9.8–11.8	0/16	0
	17	13.2	10.3–46.3	1/18	0
	stock	10.2	10.3–12.0	0/16	0

[1] Experimental period 10 weeks. Values determined beginning week 7.
[2] Fischer derived male rats, Charles River Breeding Laboratories, Wilmington, Mass. Age at start of experiment: 30 days.
[3] Experimental period 9 weeks. Values determined beginning week 6.
[4] Sprague-Dawley derived male rats, Carworth, New City, N. Y. Age at start of experiment: 27 days.
[5] Lab Blox, Wayne Division of Allied Mills, Chicago, Ill.
[6] Fischer male rats, A. R. Schmidt, Madison Wis. Age at start of experiment: 27 to 30 days.

TABLE 3

Rat strain specificity in susceptibility to vitamin K deficiency

Rat strain	Test diet	Prothrombin time		Abnormal rats	Hemorrhagic deaths
		Avg	Range		
		seconds	*seconds*		
		Exp. 1 [1]			
CDF [2]	16	11.8	9.8–19.4	1/11	0
	16 minus vitamin K	> 43.6	10.2– > 120	9/12	0
	stock [3]	10.1	9.2–10.8	0/10	0
CFE [4]	16	12.7	10.7–19.6	0/10	0
	16 minus vitamin K	11.4	10.7–12.7	0/10	0
	17	11.9	10.8–13.6	0/10	0
	17 minus vitamin K	11.3	10.2–13.1	0/10	0
	stock	10.7	9.7–12.5	0/10	0
		Exp. 2 [5]			
ARS [6]	16	12.3	11.1–20.8	2/11	0
	16 minus vitamin K	10.8	9.6–11.8	0/9	0
	17 minus vitamin K	11.1	10.3–13.8	1/10	0

[1] Experimental period: 5 weeks. Values determined during week 5 except for the stock group (weeks 7 to 9).
[2] See footnote 2, table 2.
[3] See footnote 3, table 2.
[4] See footnote 5, table 2. Age at start of experiment: 25 days.
[5] Experimental period: 9 weeks. Values determined between week 6 and 9.
[6] See footnote 6, table 2. Age at start of experiment: 25 days.

ceptibility to vitamin K deficiency have been reported (4, 5) experiment 2 was conducted to determine the susceptibility of CFE (a Sprague-Dawley-derived strain) and Fischer-strain rats (from A. R. Schmidt, designated ARS) to the condition produced by diet 17. The results (table 2) show that of the three strains tested only CDF rats had prolonged prothrombin times or died from hemorrhage. All CFE animals were normal and only one ARS rat fed diet 17 was hypoprothrombinemic.

Table 3 (exp. 1) shows differences in susceptibility to vitamin K deficiency when

chemically defined liquid diets were fed to the three different rat strains. Nine of twelve CDF rats ingesting diet 16 without menadione had prolonged prothrombin times. CFE rats fed the same diets were all normal and only three ARS rats had slightly prolonged prothrombin times. The results provided additional evidence that diet 17 produced a vitamin K deficiency in CDF rats and explained the absence of hypoprothrombinemia in CFE and ARS animals fed diet 17 in experiment 2 (table 2). In view of these strain differences, CDF male rats were used in all subsequent experiments except the one reported in table 6, where CFE rats were used.

One of the major differences between amino acid mixtures 16 and 17 is the much higher concentration of ethyl cysteinate·HCl (EC) in diet 17 (2.42 versus 0.55 g/liter). This variance is of particular interest since the —SH group of cysteine is known to react with menadione in several ways (11, 12). The effect of EC concentration on prothrombin times is reported in table 4. The results show that the hypoprothrombinemia produced by diet 17 is related to the dietary concentration of EC. At the concentration normally present in diet 17 (2.42 g/liter), and at essentially half this concentration (1.22 g/liter), prothrombin times were prolonged. When the diet was void of EC, one abnormal prothrombin time was detected.

The experiment reported in table 5 had three objectives: to determine whether EC is effective in producing the hemorrhagic syndrome when added to an amino acid mixture other than mixture 17; to determine whether chemically synthesized menadione-thioether, a possible end-product of the interaction between menadione and

TABLE 4

Effect of ethyl cysteinate·HCl concentration on the prothrombin times of rats [1]

Test diet	Ethyl cysteinate·HCl conc	Prothrombin time [2]		Abnormal rats	Hemorrhagic deaths
		Avg	Range		
	g/liter	*seconds*	*seconds*		
17	0.0	12.3	9.8–28.1	1/12	0
17	1.22	> 30.9	10.5– > 120	9/13	0
17	2.42	> 42.8	10.6– > 120	8/13	0
Stock [3]	—	12.6	10.3–15.3	0/7	0

[1] See footnote 2, table 2.
[2] Experimental period: 9 weeks. Values determined beginning week 4.
[3] See footnote 3, table 2.

TABLE 5

Effect of ethyl cysteinate·HCl, an oxygenated fat mix and menadione thioether on the prothrombin time of rats [1]

Group	Test diet	Prothrombin time [2]		Abnormal rats	Hemorrhagic deaths
		Avg	Range		
		seconds	*seconds*		
1	16	12.3	9.9–18.7	1/8	0
2	As group 1 with oxygenated fat mix	14.3	10.2–23.9	4/10	0
3	16 with 2.42 g/liter ethyl cysteinate·HCl	24.7	10.1–59.4	6/10	1
4	As group 3 with oxygenated fat mix	> 59.9	10.2– > 120	8/10	1
5	16 with 5.5 mg/liter menadione thioether	14.6	9.7–21.6	5/10	0
6	As group 5 with oxygenated fat mix	24.6	10.1–84.9	5/10	0

[1] See footnote 2, table 2.
[2] Experimental period: 9 weeks. Values determined beginning week 5.

ethyl cysteinate (13), acts as an antimetabolite of vitamin K; and to determine whether an oxygenated fat mix influences the incidence or severity of hemorrhage. It was postulated that the reactions leading to vitamin K inactivation may be catalyzed by free radical intermediates of fatty acid peroxidation.

Menadione-thioether was synthesized by modification of the method of Nickerson et al. (13). It was added to provide approximately 30% more than would be expected if all the menadione in the diet reacted with EC. The composition of the oxygenated fat mixture was the same as previously described. It was oxygenated by bubbling oxygen through the solution for 18 hours at 24–26° prior to its inclusion in the diet. When used, it replaced the standard fat mix which was always kept frozen prior to its addition to the diet.

The results show that the hypoprothrombinemia originally produced by diet 17 is not unique to this diet and can be produced by other liquid diets containing EC. As with diet 17, the incidence and severity of abnormal prothrombin times was related to EC concentration, being greatest when diet 16 contained 2.42 g/liter. The oxygenated fat mix also influenced the hemorrhagic syndrome. At both dietary levels (0.55 and 2.42 g/liter) prothrombin times and the number of abnormal animals increased when the oxygenated fat mix was used.

When menadione-thioether was added to the diet, half the rats were abnormal. The number of abnormalities was the same regardless of the use of the oxygenated fat mix, although in the latter instance the range of prothrombin times was elevated. It was of interest that feeding much higher dosages (15 mg/liter) of this adduct to male CFE rats was without effect (table 6). There was no evidence of antivitamin K activity even when vitamin K-free diets were fed and the only source of this vitamin was from intestinal synthesis. All animals were normal and in no instance were prothrombin times prolonged.

In view of these findings, experiments were conducted to determine the specificity of the EC–menadione interaction, and to determine whether ethyl linoleate, as a major fatty acid and the most labile component of the fat mix plays a role in accelerating the hemorrhagic condition.

To test the specificity of menadione an equimolar amount of Synkavite [10] (menadiol sodium diphosphate) was added to the diet. This compound was selected because the 1- and 4-positions are occupied by the phosphate esters and the 3-position is no longer an active site for ethyl cysteinate addition due to the reduced state of the quinone nucleus. The specificity of EC was tested by replacing it with diethyl cystinate·2HCl. The configuration of this molecule is such that free —SH groups are not available for attachment at the 3-position of the menadione molecule.

The data in table 7 show that hypoprothrombinemia is specific for menadione and EC, and can be prevented by either substituting Synkavite for menadione or cystine diethylester·2HCl for EC. Seven of eight rats had prolonged prothrombin times when EC and menadione were present in the diet. No abnormalities occurred, however, when they were replaced by diethyl cystinate or Synkavite.

[10] Synkavite: Trademark for menadiol sodium diphosphate, a product of Roche Laboratories, Nutley, N. J.

TABLE 6

Effect of menadione thioether on the prothrombin time of CFE rats [1]

Test diet	Prothrombin time [2]		Abnormal rats	Hemorrhagic deaths
	Avg	Range		
	seconds	*seconds*		
16+5 mg/liter menadione thioether	10.9	10.1–12.7	0/9	0
16+5 mg/liter menadione thioether minus vitamin K	10.7	9.7–12.8	0/9	0
16+15 mg/liter menadione thioether	10.5	9.7–11.7	0/9	0

[1] See footnote 4, table 2.
[2] Experimental period: 9 weeks. Values determined beginning week 7.

TABLE 7

Effect of substituting diethylcystinate·2HCl for ethyl cysteinate·HCl and menadiol sodium diphosphate for menadione on the prothrombin time of rats [1]

Test diet	Prothrombin time [2]		Abnormal rats	Hemorrhagic deaths
	Avg	Range		
	seconds	*seconds*		
17	29.3	11.1–38.3	7/8	0
17 with menadiol sodium diphosphate [3]	10.8	9.8–12.1	0/8	0
17 with diethyl cystinate·2HCl	10.4	9.6–11.3	0/8	0

[1] See footnote 2, table 2. Age at start of experiment: 36 days.
[2] See footnote 2, table 5.
[3] Synkavite, Roche Laboratories, Nutley, N. J.

TABLE 8

Effect of ethyl linoleate on the prothrombin time of rats [1]

Test diet	Prothrombin time [2]		Abnormal rats	Hemorrhagic deaths
	Avg	Range		
	seconds	*seconds*		
17	> 73.3	12.6– > 120	14/15	6
17 minus ethyl linoleate	34.2	10.4–80.4	13/15	1
17 minus ethyl cysteinate·HCl	10.7	9.8–12.3	0/15	0
17 minus ethyl linoleate and ethyl cysteinate·HCl	10.8	9.8–11.8	0/15	0

[1] See footnote 2, table 2.
[2] Experimental period: 9 weeks. Values determined beginning week 7.

TABLE 9

Effect of dl-α-tocopheryl acetate and ascorbic acid on the prothrombin time of rats [1]

Group	Test diet	Prothrombin time		Abnormal rats	Hemorrhagic deaths
		Avg	Range		
		seconds	*seconds*		
		Exp. 1 [2]			
1	17	22.9	17.8–32.3	10/10	0
2	17 + vitamin C + vitamin E [3]	> 88.9	26.7– > 120	10/10	6
3	As group 2 minus ethyl linoleate	35.8	17.6–72.1	10/10	6
		Exp. 2 [2]			
1	17 + vitamin E	21.3	12.2–33.7	3/5	0
2	17 + vitamin C	18.3	9.8–35.3	3/5	1
3	17 minus vitamin A	20.9	9.3–26.5	3/4	0
4	As group 3 + vitamin E	23.6	9.8–48.3	3/5	2
5	As group 3 + vitamin C	34.3	16.1–45.6	4/5	1
6	17 minus EC [4]	11.1	9.5–13.5	0/5	0
7	As group 6 minus vitamin A	11.2	9.8–14.6	0/5	0

[1] See footnote 2, table 2. Age at start of experiments 1 and 2, 51 days and 40 to 42 days, respectively.
[2] Experimental period: 7 weeks. Values determined beginning week 3.
[3] Two hundred fifty milligrams per liter supplemental vitamin C (ascorbic acid); 25 mg/liter supplemental vitamin E (*dl-α*-tocopheryl acetate).
[4] Ethyl cysteinate·HCl.

The effect of ethyl linoleate on the hemorrhagic syndrome is shown in table 8. The results show that the syndrome only occurred when EC was present in the diet (2.42 g/liter) whether or not ethyl linoleate was also present. Ethyl linoleate seemed to influence the severity but not the incidence of the condition. Prothrombin times were longer and the number of hemorrhagic deaths (6 versus 1) greater when it was

present in the diet. However, no deaths or hypoprothrombinemia occurred when the diet was void of EC.

The influence of antioxidants on the severity of the hemorrhagic syndrome is reported in table 9. The antioxidants studied were ascorbic acid and *dl*-α-tocopheryl acetate. They were added to the diet at twice their ordinary concentrations (i.e., 500 and 50 mg/liter, respectively).

The results show that supplementation with these antioxidants did not inhibit the hemorrhagic syndrome characteristically produced by diet 17. In fact, their addition appeared to increase its severity even when ethyl linoleate was excluded from the diet.

A second experiment (table 9) was conducted to determine the influence of ascorbic acid and *dl*-α-tocopheryl acetate when added to the diet separately, and to determine whether vitamin A is also involved in enhancing the severity of the hemorrhagic syndrome. Matschiner and Doisy (14) have shown that physiological levels of vitamin A enhance hypoprothrombinemia in rats fed diets low in vitamin K. It was hypothesized that ascorbic acid and *dl*-α-tocopheryl acetate may protect vitamin A and enable it to act in its hypoprothrombinemic capacity. The results show that at twice their ordinary concentrations, separate supplements of ascorbic acid and *dl*-α-tocopheryl acetate did not influence the incidence or severity of the hemorrhagic syndrome. This was true regardless of the presence of vitamin A in the diet. As observed earlier, the exclusion of EC from the diet completely prevented the syndrome. Neither the simultaneous addition of ascorbic acid and *dl*-α-tocopheryl acetate, nor the exclusion of vitamin A from the diet, influenced prothrombin times as long as EC was not present.

DISCUSSION

It is clear from these results that ethyl cysteinate·HCl (EC) is the primary factor responsible for the hemorrhagic syndrome in rats ingesting chemically defined liquid diets containing menadione. In virtually all cases where EC was absent from the diet, rats were normal and no prolonged prothrombin times were observed (tables 4, 8 and 9). When EC was present, the incidence and severity of the syndrome was related to its concentration in the diet (tables 4 and 5).

Reports in the literature have shown that cysteine prolongs blood coagulation time when added to whole blood or when administered orally or intravenously to human subjects (15, 16). Sterner and Medes (16) concluded that it prevents the activation of prothrombin to thrombin. Although the possibility of a humoral effect cannot be ruled out, it is more likely that in the present study EC produces hypoprothrombinemia indirectly, by causing a dietary vitamin K deficiency.

The underlying mechanism responsible for this condition was found to be the interaction of the free thiol groups of EC with menadione. This was demonstrated when the hemorrhagic syndrome was completely prevented by replacing EC with an equimolar amount of diethyl cystinate·2HCl (table 7). The structure of diethyl cystinate·2HCl is such that there are no free thiol groups available for interaction with menadione.

Menadione can react with thiols in several ways: addition of the −SH group to the reactive double bond of the quinone; formation of insoluble polymers; reaction of the −SH groups with the two carbonyl groups of the quinone (12); interaction at the 3-position of the quinone nucleus to yield a quinone thioether, i.e., 2-methyl, 3-cysteinyl, 1,4-naphthoquinone (13). The structure of the quinone is important in determining which of these reactions takes place.

Replacing menadione with an equimolar amount of menadiol disodium phosphate completely prevented the hemorrhagic syndrome (table 7). The 1-, 4-positions of this molecule are occupied by sodium diphosphate and the 3-position is no longer an active site for EC addition due to the reduced state of the quinone nucleus. Undoubtedly, at least one of these positions is involved in the interaction between EC and menadione. Our present knowledge, however, does not permit us to decide the primary site of action or the predominant reaction mechanism.

Under the conditions of these experiments the fat components played a secondary role in producing hypoprothrombinemia. Oxygenation of the fat mix increased the severity of the hemorrhagic condition

slightly. Removal of ethyl linoleate, the primary source of unsaturation, reduced the severity. These effects were not observed in the absence of EC, suggesting that the fat components indirectly influenced the hemorrhagic condition by modifying the interaction between EC and menadione. The regulating mechanism may be related to the formation of free radicals resulting from the peroxidation of the unsaturated fatty acids in the diet.

Matschiner and Doisy have also reported on the hemorrhagenicity of oxidized lipids fed to rats.[11] They found that oxidized soybean oil or neutral beef fat were hypoprothrombinemic, whereas the methyl esters of oleate and linoleate were ineffective after oxidation. The oleate moiety of polysorbate 80 and ethyl linoleate were the only fatty acids present in our fat mix. The moderate response to these fatty acids in the present study may be related to their secondary role and to differences in the physical form and chemical composition of our diets.

The susceptibility of EC to auto-oxidation may also have influenced the results of these studies. Since cystine ethyl ester, the oxidized form of EC, does not have free thiol groups available for interaction with menadione, the extent of vitamin K deficiency and consequently the degree of hemorrhagenicity, could have been dictated by the rate and extent of its formation. The pH of our diets varied between 4.7 and 5.0. Under these conditions, auto-oxidation is not very rapid, even in the presence of the many metallic salts found in the diet. Exposure to air for any length of time, however, causes slow conversion of the reduced form of EC to the equally soluble cystine ethyl ester. This conversion may have taken place when the diets were in the Richter tubes and during refrigeration. Even though all bottles were stoppered during refrigeration their daily exposure to the atmosphere at feeding time may have provided sufficient air to initiate auto-oxidation.

It was suggested that menadione thioether (2-methyl, 3-cysteinyl, 1,4-naphthoquinone), an adduct of the interaction between the thiol group of EC and the 3-position of menadione, might act as an antimetabolite of vitamin K and thereby contribute to the observed hypoprothrombinemia. The results of feeding diets containing this compound are equivocal. Half the CDF rats fed this additive were abnormal (table 5). On the other hand, CFE rats receiving much higher concentrations of the thioether were all normal (table 6). The results obtained with CDF rats may have been complicated by the presence of EC (0.55 g/liter) in the basal diet. This level of EC was found to be borderline in our other experiments with this strain, and its presence in the diet could have contributed to the observed response. CFE rats were fed the same basal diet; however, this strain was found to be more resistant to vitamin K deficiency (table 3), and consequently, it is doubtful in this case that the presence of EC influenced the results. Indeed, the fact that these rats remained normal when consuming diets containing almost three times the concentration of menadione thioether fed to the CDF animals, suggests that although it may be a product of menadione destruction, it does not act as an antimetabolite.

Differences in strain susceptibility to vitamin K deficiency and to the hemorrhagic condition produced by EC were observed in these studies (table 3). Sprague-Dawley-derived CFE rats [12] and Fischer-derived ARS rats [13] were resistant to vitamin K deficiency, whereas Fischer-derived CDF rats [14] were highly susceptible. Strain differences in susceptibility to vitamin K deficiency have been reported before (4, 5). In the present case, however, rats derived from the same strain but purchased from different sources were markedly different in their susceptibility. This points up the importance of considering species origin as well as strain when evaluating experiments of this type and when selecting animals.

The severity of the hemorrhagic syndrome when ascorbic acid and *dl*-α-tocopheryl acetate were simultaneously added to the diet at twice their ordinary concentrations (table 9) suggests that they were protecting EC in their capacity as antioxidants. This view is supported by the fact

[11] Matschiner, J. T., and E. A. Doisy, Jr. 1961 The hemorrhagenicity of oxidized lipids. Federation Proc., 20: 365 (abstract).
[12] See footnote 4.
[13] See footnote 5.
[14] See footnote 3.

that these vitamins were ineffective in the absence of EC. The failure of the same concentration of either vitamin alone to affect the hemorrhagic condition indicates an enhanced antioxidant effect when they were present together. It is known that ascorbic acid and tocopherols form powerful synergistic antioxidant combinations (17).

Ascorbic acid and vitamin E did not exert their effect through protection of vitamin A as originally postulated. There was no measurable hemorrhagic response to the elimination of vitamin A acetate from the diet (table 9). The mechanism by which vitamin A participates in the development of vitamin K deficiency is not fully understood and conflicting reports on its hemorrhagenicity appear in the literature. Our results are in accordance with those of Wostmann and Knight (18) who did not observe any antagonism between vitamins A and K at physiological doses (5 to 50 IU/day). They are in disagreement, however, with the results of Matschiner and Doisy (14) who reported that 0.5 to 5 IU vitamin A/g of vitamin K-deficient diet reduced the prothrombin concentrations of rats.

Finally, attention should be given to the function of the physical form of the diet in precipitating the conditions reported here. Clearly, susceptibility of dietary ingredients to interaction is much greater in solution than in the dry solid state. Furthermore, fecal output of rats ingesting liquid diets of the type used in these studies is markedly reduced. Observations in our laboratory [15] showed more than a 90% reduction in fecal output when switched from a commercial ration to a chemically defined liquid diet. This reduction could be of importance to the rat who can prevent vitamin K deficiency through coprophagy. In this connection, it is noteworthy that chemically defined liquid diets have caused dramatic alterations in the intestinal flora of human subjects.[16] Such changes might also occur in rats, especially if coprophagy is reduced. This, in turn, could influence the extent of bacterial synthesis of vitamin K in the intestine. It would be of interest to determine whether solid, dry diets of the same composition used in these studies can produce the hemorrhagic syndrome reported here.

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Absorption, Deposition and Placental Transfer of Sulfate Sulfur by Gilts [1]

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ABSTRACT Seventeen swine were dosed with sodium ^{35}S sulfate to study the absorption, excretion, placental transfer and maternal-fetal tissue distribution of sulfur. During 7-day balance studies, barrows consumed 2.21 g sulfur/day and excreted 1.31 and 0.40 g sulfur/day in urine and feces, respectively. Both orally and intravenously administered ^{35}S were rapidly eliminated primarily through urinary excretion. Blood levels decreased 90% within 24 hours after intravenous dosing, and ^{35}S administered orally reached a peak level in whole blood and serum after 3 hours, after which time the concentration curves reflected a gradual disappearance rate to 168 hours, similar to that for ^{35}S injected. Data obtained from gilts killed at 38, 76 and 112 days gestation revealed that most maternal tissues contained between 1 and 2 mg S/g fresh tissue, compared with less than 1 mg/g for fetal tissue. In the products of conception, the proportion of ^{35}S decreased in the placenta and fluids and increased in fetuses as pregnancy advanced. Labeled sulfur concentration was greater in bone than soft tissues in both maternal and fetal tissues. Six hours after intravenous dose administration ^{35}S concentration in maternal tissue was highest, then decreased with time, whereas fetal tissues did not obtain maximum ^{35}S levels until after 24 hours and receded progressively during the following 13 days. Gilts of trimester 3 deposited, after 7 days, 9% of that ^{35}S retained in the fetuses (64%), the uterus and maternal placenta (26%) and the fluids (10%). Because increasing increments of ^{35}S occurred in the fetus concurrent with advancing pregnancy, placental transfer appeared to be a direct function of need or fetal age.

Elemental sulfur can be incorporated into sulfur-containing amino acids by rumen bacteria (1–6), and though this process cannot be accomplished in mammalian tissues (7, 8), inorganic sulfur can be used to meet part of the monogastric animal's dietary requirement for use in such sophisticated processes as sulfation of cartilage mucopolysaccharides (9), and formation of sulfolipids and other mammalian sulfate esters (10–12). The metabolism of sulfur by organisms has been extensively investigated from various nutritional and biochemical aspects. Through these studies the beneficial value of supplemental inorganic sulfur to ruminants fed high nonprotein nitrogen rations (13–15), the mechanisms involved in assimilatory sulfate reduction by microorganisms [4] (16) and the reactions associated with sulfate activation (17, 18) have been established. The role of sulfur in fetal nutrition, however, has not been well documented.

Previous reports have demonstrated that sulfur is rapidly transferred to the fetuses of rats (19), cattle (20) and sheep (21), and that the placenta of swine is readily permeable to iron (22), calcium (23) and zinc (24). This investigation was concerned with the absorption, excretory patterns and the maternal-fetal tissue distribution of sulfur, and with quantitating sulfur movement through and within the gravid gilt at three trimesters of pregnancy.

EXPERIMENTAL PROCEDURE

Fifteen gilts and two barrows of mixed breeding with an average weight of 145 and 90 kg, respectively, were fed 1800 g/day of a conventional corn-soybean meal ration containing 0.63% calcium, 0.48% phosphorus and 0.14% sulfur for an ap-

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proximately 4-week preliminary period and throughout the balance and placental transfer studies. The gilts were placed in metabolism crates and dosed intravenously with 5 mCi carrier-free $Na_2{}^{35}SO_4$ 7 days prior to termination of trimesters 1, 2 or 3 of pregnancy. At 168 hours postdosing, 10 animals were killed; the uteruses were removed intact and the products of conception (fluids, membranes and fetuses) were weighed and sampled separately. Five trimester 3 gilts were slaughtered at 6, 24, 48, 120 and 336 hours after dosing for kinetic comparison with those killed at 168 hours. About half of the fetuses from each litter were sampled for individual tissue analyses and half were ashed for whole-body analyses. Fetuses from trimesters 2 and 3 were homogenized in a heavy-duty stainless steel blender[5] to allow for sampling and whole-body analyses for total sulfur and ^{35}S. An additional 20 gravid gilts were killed during trimesters 2 and 3 for total sulfur concentrations in maternal and fetal tissue. The number of swine used at each stage of gestation, and the time interval after dose administration is tabulated in tables 1, 2 and 5. Two barrows were placed in metabolism units equipped to allow separate collections of urine and feces, and dosed either orally or intravenously with labeled sulfate at five separate times. Blood samples and excreta were obtained from all animals at expanding time intervals after dosing to 7 days.

Excretory, blood and tissue samples were digested in a 2:1 nitric–perchloric acid mixture as outlined by Eldjarn and Nygaard (25), and sulfur and ^{35}S assayed by $BaSO_4$ precipitation according to the procedure of Katz and Golden (26) with the modification described by Hansard and Mohammed (21). Sulfur-35 counting was performed on a low-background, gas-flow beta-detector. For comparative purposes all maternal tissue ^{35}S values were adjusted to 150 kg live body weight and litter size was standardized to eight fetuses weighing 5, 350 and 1,000 g at trimesters 1, 2 and 3 of pregnancy, respectively. Balance data were used for calculating quantity of the *retained* ^{35}S dose in the various tissues and organs.

RESULTS AND DISCUSSION

Blood sulfur-35. The level of ^{35}S in blood of orally or intravenously dosed swine as a function of time after isotope administration is presented in figure 1. Hematocrits and separate analyses of packed cells and serum were used to determine blood ^{35}S values. Intravenously administered ^{35}S rapidly disappeared from the blood as concentration values decreased to half their initial level within 3 hours after dosing, and continued to decline sharply to less than 10% after 24 hours postdosing. Between 24 and 168 hours after dosing, ^{35}S rate of removal was low and values decreased progressively to less than 1% of the administered dose. Labeled sulfur given orally was quickly absorbed by swine, and was detected in the blood within 30 minutes after dose administration. After 3 hours, ^{35}S activity reached a peak level in both blood and serum, then disappearance rate paralleled that of the intravenous ^{35}S during the following 165 hours. Serum contained slightly more ^{35}S per unit volume than erythrocytes during the first 24 hours, after which time the radiosulfur tended to become equally distributed between the two fractions.

Sulfur and ^{35}S excretion. In five separate 7-day blood-balance studies, two barrows fed rations containing 0.14% sulfur consumed 2.21 ± 0.18 g sulfur/day and excreted 1.31 ± 0.09 and 0.40 ± 0.04 g sulfur/day in urine and feces, respectively. Results from this study reveal a greater proportion of excretory sulfur to be eliminated in the urine by swine than by cattle (20) or sheep (21) fed dietary regimes comparable in sulfur content. Employing procedures which have shown that sulfur consumed in excess of dietary requirements is excreted in the urine, Wellers and coworkers (27) were able to demonstrate the existence of a nutritional sulfur balance dependent on sulfur consumption. Numerous studies which revealed that ruminants and nonruminants utilize inorganic sulfur differently may partially explain the difference in sulfur excretory patterns observed among species.

Labeled sulfur, administered either orally or intravenously to swine, was

[5] Waring Blendor, Waring Products Company, Winsted, Conn.

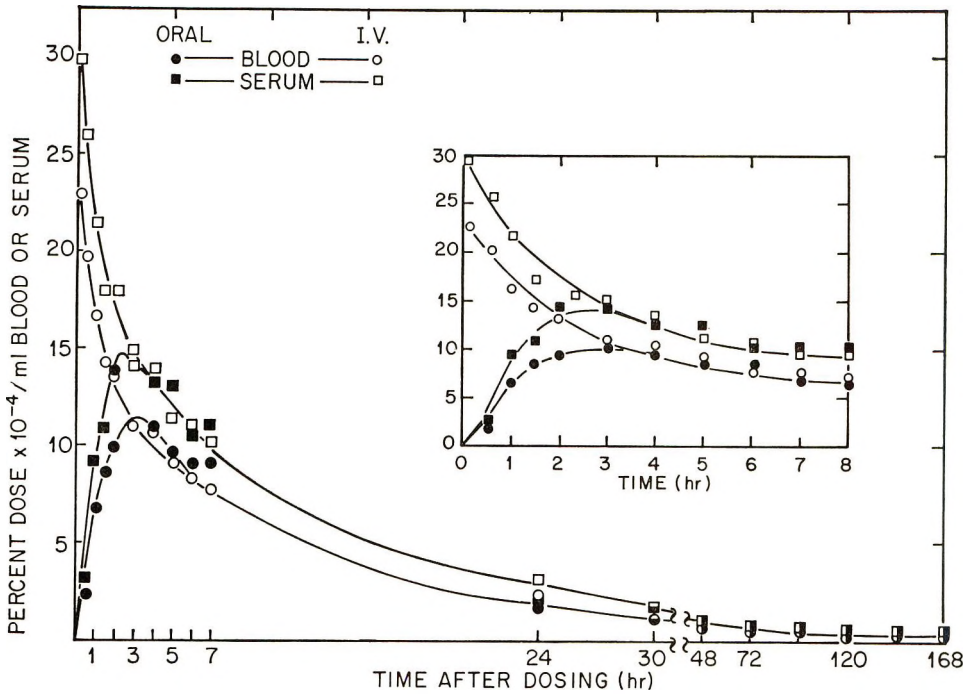

Fig. 1 Changes in blood and serum ^{35}S levels in swine following oral and intravenous administration of labeled sulfate. These composition relationships are further emphasized in the expanded insert as a function of time.

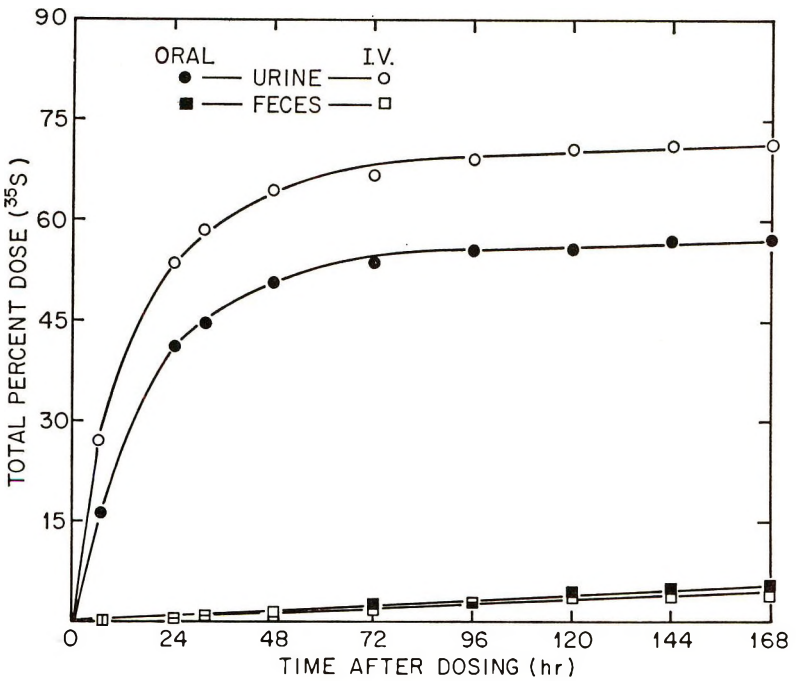

Fig. 2 Accumulative urinary and fecal excretion of orally and intravenously administered ^{35}S by swine.

rapidly removed from the body through urinary elimination (fig. 2). During a 7-day period, urinary and fecal excretion accounted for 72 and 4% of that intravenously administered, and for 58 and 5% of the oral ^{35}S, respectively. Within 6 hours after intravenous dosing, 27% of the labeled sulfur had been removed through urinary excretion. Differences in elimination of ^{35}S in urine due to route of isotope administration occurred within 24 hours after dosing. Similar ^{35}S excretory patterns have been reported to exist for sheep and cattle by Hansard and Mohammed (20, 21). Dogs (28), bullocks (29) and man (30) also eliminate sulfate from the body principally via urinary excretion. According to Bourdillon and Lavietes (30) the rate of urinary sulfate excretion appears to be simply proportional to the concentration of sulfate in serum. Goudsmit et al. (28), however, reported the reabsorption of sulfate from glomerular filtrate to vary directly with sulfate concentration in the blood. Thus, blood sulfate levels, through influence on renal filtration and reabsorption processes, would exert a definite effect on kidney sulfate clearance.

Results obtained from the chemical studies closely paralleled those from the labeled sulfate, and demonstrated sulfate absorption in swine to be relatively high. Sulfur absorption is also apparently somewhat dependent upon dietary form as Denis and Reed (31) and Kellermann (32) report flowers of sulfur to be poorly ($< 30\%$) absorbed by rats and sheep, whereas other studies have indicated a rapid and high absorption of labeled sulfate in cattle (20), sheep (21) and swine (33). Reasons for this rapid absorption and subsequent rapid urinary elimination of sulfate, as well as the utilization differences observed between inorganic to organic sulfur by swine, needs further investigation to evaluate accurately their usefulness to the animal.

Maternal and fetal tissue sulfur concentrations. The data in table 1 show the concentration of sulfur in maternal and fetal tissues at three trimesters of pregnancy. As stage of gestation had no detectable influence on maternal tissue sulfur

TABLE 1

Influence of advancing pregnancy on maternal and fetal tissue sulfur concentration in swine [1]

Tissue	Maternal	Fetal, trimester		
		1 (4) [2]	2 (8)	3 (23)
Blood	1.04 ± 0.03 [3]		0.43 ± 0.03	0.43 ± 0.03
Serum	0.88 ± 0.02		0.31 ± 0.08	0.22 ± 0.02
Liver	2.24 ± 0.06	1.26 ± 0.14	1.16 ± 0.07	1.03 ± 0.02
Kidney	1.83 ± 0.06		0.67 ± 0.05	0.89 ± 0.03
Spleen	1.87 ± 0.05		0.89 ± 0.10	0.77 ± 0.03
Heart	1.92 ± 0.06		1.05 ± 0.10	0.99 ± 0.03
Muscle	1.89 ± 0.08	0.97 ± 0.47	0.62 ± 0.03	0.93 ± 0.02
Skin	1.13 ± 0.07		0.36 ± 0.02	1.25 ± 0.05
Aorta	1.28 ± 0.07		0.64 ± 0.10	0.81 ± 0.04
Brain	1.27 ± 0.08		—	0.85 ± 0.05
Pancreas	1.74 ± 0.08		0.75 ± 0.04	1.17 ± 0.05
Pituitary	1.37 ± 0.13		—	1.23 ± 0.21
Whole fetus	—	0.60 ± 0.06	0.77 ± 0.04	1.01 ± 0.02
Adrenal	1.65 ± 0.05		—	—
Bile	0.75 ± 0.12		—	—
Mammary tissue	1.23 ± 0.13		—	—
Mandible	0.76 ± 0.08		0.67 ± 0.09	0.58 ± 0.02
Sternum	1.11 ± 0.14		1.62 ± 0.13	1.68 ± 0.06
Rib shaft	0.74 ± 0.04		1.04 ± 0.13	0.73 ± 0.04
Rib end	1.08 ± 0.07		1.48 ± 0.35	0.97 ± 0.06
Femur shaft	0.63 ± 0.07		0.78 ± 0.10	0.68 ± 0.03
Femur end	1.23 ± 0.15		1.59 ± 0.09	0.91 ± 0.04

[1] Concentration values expressed as milligrams sulfur per gram fresh tissue.
[2] Refers to number of gilts killed at each trimester.
[3] ± SE of the mean.

levels, all values were pooled for ease of presentation. Maternal liver, heart, muscle, spleen and kidney contained approximately 0.20% sulfur as compared with sulfur levels ranging between 0.06 and 0.13% for most other maternal tissues analyzed. Although blood and serum values for these gilts are greater than those reported in the literature for most species, they are in agreement with those reported by Kellermann (34) for young and mature rats fed three levels of sulfur. Upon equilibration to fresh tissue weight, rat soft tissue values, ranging from 7 to 11 mg/g dry matter (34), are similar to values observed for swine. The soft tissue of gilts contained a greater concentration (~ twofold) of sulfur than corresponding fetal tissue, whereas similar levels of sulfur were detected in maternal and fetal bone. Sulfur tended to be equally distributed between soft and hard tissues in fetuses. With the exception of fetal skin which increased threefold during the last trimester of pregnancy, fetuses of trimesters 2 and 3 contained analogous concentrations of sulfur in most tissues. Analyses of whole fetuses, however, indicated that total fetal sulfur levels increased during gestation to 0.10% sulfur near term.

Sulfur and ^{35}S in products of conception. Accretion of stable and labeled sulfur, 168 hours after dosing, occurred in all conception products between trimesters 1 and 2, and in the uterus, maternal placenta and fetuses during trimesters 2 and 3 of pregnancy (table 2). Studies by Feaster and Davis (19) showed that the amount of ^{35}S transferred to the rat fetus in 48 hours is elevated as fetal age increased. Quantity of ^{35}S in the placental fluids was much greater at the end of trimester 2 than trimester 1; however, due to a change in fluid volume, values decreased toward the end of pregnancy. During trimester 1, most of the sulfur was deposited in the uterus and maternal placenta. Total ^{35}S contained in the whole uterine complex at this time was 0.65% of the retained ^{35}S dose, and was partitioned as follows: 90% in the uterus and maternal placenta; 8% in fluids; and 2% in total fetuses. At the end of trimester 2, fetuses contained nearly as much ^{35}S and 50% less total sulfur than both the uterus and placenta. The quantity of sulfur and ^{35}S in trimester-3 fetuses was twofold that detected in the remaining products of conception. Eight of these fetuses standardized to 1000 g, contained 8.11 g sulfur and after 7 days, 5.75% of that ^{35}S retained had been transferred to the fetuses.

Maternal and fetal tissue ^{35}S concentration as affected by gestation age. The concentration of ^{35}S in selected maternal and fetal tissue at three trimesters of gestation is shown in table 3. Stage of pregnancy had no appreciable influence on maternal or fetal tissue ^{35}S levels. Dams and fetuses contained greater concentrations of ^{35}S in bones than soft tissues. Concentration of labeled sulfur was greatest in bone areas associated with most active mineral deposition and mobilization. Observations on the deposition of ^{35}S in the tibiae of rabbits (35) and long bones of rats (36) obtained from use of chemical and chromatographic techniques revealed

TABLE 2

Effect of gestation age on sulfur and ^{35}S in conception products of swine [1]

Product	Gestation age, days					
	38 (4) [2]		76 (2)		112 (4)	
	Sulfur	^{35}S	Sulfur	^{35}S	Sulfur	^{35}S
	g	*%*	*g*	*%*	*g*	*%*
Uterus and maternal placenta	1.60	0.47	5.47	2.05	5.75	2.37
Placental fluids	0.12	0.17	0.36	1.13	0.22	0.88
Whole fetuses [3]	0.03	0.01	2.16	1.74	8.11	5.75
Total	1.75	0.65	7.99	4.92	14.14	9.00

[1] Total sulfur and percentage retained ^{35}S at 168 hours after dose administration to 150-kg gilts.
[2] Number of gilts killed at each trimester of pregnancy.
[3] Litter size standardized to eight fetuses with a weight of 5, 350 and 1000 g at 38, 76 and 112 days gestation, respectively.

that ^{35}S, other than dialyzable inorganic sulfate, was primarily incorporated into chondroitin sulfate. Fetal bones contained a greater concentration of ^{35}S than maternal bones, and fetal sternum, rib end and femur end had notably more ^{35}S than other tissue analyzed. Labeled sulfur concentration was higher in soft tissues of gilts than in fetuses. Kulwich et al. (33), through studies concerned with the metabolic fate of orally administered labeled sulfate to baby pigs, discovered aorta to have the highest concentration and muscle and brain the lowest concentration of ^{35}S, which is in agreement with soft tissue ^{35}S values for older swine and fetuses.

Partition of ^{35}S between gilt and fetal organs. The total percentage retained ^{35}S in maternal and fetal liver, kidney, heart and spleen at 38, 76 and 112 days gestation, 168 hours after intravenous administration, is presented in table 4. Gilts killed at 78 and 112 days gestation contained more ^{35}S in selected organs than those killed 38 days following conception. The magnitude of difference between the total quantity of ^{35}S deposited in organs of gilts and their fetuses, standardized to eight per litter, was due to a difference in organ size rather than the ^{35}S concentration. Due to an enlargement in fetal organ weight, the quantity of ^{35}S deposited in these organs

TABLE 3

Gestation age effects upon maternal and fetal tissue ^{35}S concentration in swine [1,2]

Tissue	Trimester					
	1		2		3	
	Dam	Fetus	Dam	Fetus	Dam	Fetus
Liver	1.83	1.29	2.46	1.71	2.25	1.71
Kidney	3.25	3.16	4.71	2.08	4.42	3.50
Spleen	3.42		4.83	2.17	4.33	2.42
Heart	1.08	1.33	2.00	2.62	1.70	1.75
Muscle	0.29	1.62	0.58	2.42	0.29	1.62
Skin	4.46		6.00	2.75	3.29	4.12
Aorta	6.08		8.00	5.54	8.25	6.20
Brain	0.67		0.29	1.12	0.83	1.83
Pancreas	1.00		1.08	2.17	1.25	1.96
Pituitary	3.88		4.46	2.50	4.95	2.45
Whole fetus		2.37		6.25		7.21
Mandible	3.42		2.50	10.08	3.58	6.46
Sternum	7.62		8.08	46.79	8.58	36.96
Rib shaft	5.58		4.83	10.12	4.58	7.33
Rib end	31.12		33.83	43.16	25.12	21.67
Femur shaft	1.88		2.62	7.88	2.08	5.83
Femur end	15.71		15.54	52.58	7.04	18.29

[1] Concentration values are calculated as percentage retained $^{35}S \times 10^{-4}/g$ after 168 hours in 150-kg gilts.
[2] Number of gilts killed at each trimester of pregnancy is listed in table 2.

TABLE 4

Total quantity of ^{35}S in maternal and fetal organs of swine at three trimesters of pregnancy [1]

Trimester	Organ							
	Liver		Kidney		Heart		Spleen	
	Dam	Fetus [2]	Dam	Fetus [2]	Dam	Fetus [2]	Dam	Fetus [2]
1	31.20	0.05	5.37	0.003	4.46	0.01	7.83	—
2	41.90	1.56	7.75	0.31	8.33	0.43	10.17	0.06
3	38.30	4.29	7.29	1.22	7.08	1.00	9.17	0.22

[1] Expressed as percentage retained $^{35}S \times 10^{-2}$/organ after 168 hours, corrected to 150-kg gilts.
[2] Total quantity of ^{35}S in fetal organs standardized to eight fetuses.

TABLE 5

Effect of time after dosing on ^{35}S concentration in maternal and fetal tissue of trimester-3 gilts[1]

Tissue	Hours after dose											
	6 (1) [2]		24 (1)		48 (1)		120 (1)		168 (4)		336 (1)	
	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus
Blood, ml	9.98	0.40	3.44	1.57	0.57	0.68	0.57	0.95	0.33	0.68	0.40	0.42
Liver	9.81	0.25	2.61	0.81	1.90	0.62	1.04	0.52	0.67	0.42	0.34	0.32
Kidney	12.24	0.37	2.45	1.11	2.07	0.81	2.06	0.98	1.06	0.84	0.50	0.54
Spleen	6.69	0.26	3.47	0.83	2.21	0.63	1.62	0.79	1.04	0.58	0.59	0.53
Heart	4.53	0.20	0.51	0.68	0.71	0.50	0.34	0.42	0.41	0.42	0.22	0.35
Muscle	1.82	0.19	0.35	0.62	0.21	0.48	0.08	0.36	0.08	0.37	0.06	0.35
Skin	5.60	0.42	5.29	1.39	2.91	0.94	2.45	1.32	0.79	0.99	0.42	0.85
Pancreas	4.00	0.29	1.87	0.53	0.68	0.48	0.40	0.60	0.30	0.50	0.22	0.41
Pituitary	6.90	0.18	3.91	0.66	3.32	0.60	0.92	0.90	1.18	0.59	0.70	0.56
Aorta	7.38	0.35	4.29	1.08	2.19	1.00	2.90	2.22	1.98	1.49	1.61	1.30
Brain	0.65	0.06	0.65	0.42	0.24	0.73	0.26	0.53	0.20	0.44	0.15	0.50
Whole fetus	—	0.28	—	1.81	—	1.24	—	1.90	—	1.73	—	1.64
Adrenal	8.03	—	4.82	—	1.61	—	2.40	—	1.28	—	0.66	—
Bile	21.07	—	30.20	—	14.12	—	6.42	—	0.82	—	0.28	—
Mammary tissue	6.89	—	2.25	—	2.66	—	1.46	—	0.65	—	0.33	—
Mandible	3.75	0.52	1.78	2.48	1.68	1.65	0.38	1.43	1.11	1.55	1.02	1.68
Sternum	4.47	0.48	2.10	2.26	1.94	5.36	2.25	9.45	2.06	8.87	0.67	7.61
Rib shaft	7.89	0.63	2.88	2.21	2.18	1.59	1.29	2.18	1.10	1.76	0.97	1.77
Rib end	17.98	0.83	10.22	2.76	8.15	4.10	10.40	4.56	6.03	5.20	4.93	4.26
Femur shaft	2.05	0.55	3.29	2.30	0.80	1.42	0.70	1.81	0.50	1.40	0.38	1.05
Femur end	4.62	0.53	3.70	2.71	3.68	1.93	1.94	2.87	1.68	4.39	1.79	2.10

[1] Concentration values are calculated as percentage intravenously administered dose $^{35}S \times 10^{-4}$/g, corrected to 150-kg gilts.
[2] Number of gilts killed at each time interval after dose.

increased tremendously as pregnancy advanced. Percentage increase in quantity deposited in fetal organs was greater between trimesters 1 and 2. The largest numerical increase, however, occurred during trimesters 2 and 3. Labeled sulfur was quantitatively deposited in fetal organs in the following order throughout pregnancy: liver > heart > kidney > spleen.

Kinetic movement of ^{35}S *in maternal-fetal tissues.* The data in table 5 illustrate ^{35}S concentrations in maternal and fetal tissues from trimester-3 gilts at variable time intervals after intravenous dosing. Concentrations in maternal tissue were highest after 6 hours, decreased rapidly between 6 and 24 hours, and then gradually diminished to 336 hours after dose administration. Within 6 hours after intravenous administration, substantial quantities of ^{35}S had crossed the placenta and by 24 hours, whole fetuses and most individual fetal tissues demonstrated highest concentrations of ^{35}S. Although levels of ^{35}S remained relatively constant between 24 and 336 hours following dose administration, a slight decrease in values was observed in most fetal tissue during weeks 1 and 2. After approximately 48 hours, maternal and fetal blood reached equilibrium. Fetal blood ^{35}S levels approached that in tissues, and appeared to peak after 24 hours. With the exception of muscle, which reached equilibrium before 24 hours, most of the maternal and fetal soft tissues reached ^{35}S equilibrium between 120 and 168 hours following dosing. Equilibrium time between maternal and corresponding fetal bone occurred faster than most soft tissue and approximated 24 hours. Results obtained from animals killed after 24, 168 and 336 hours indicated ^{35}S to be in a constant state of flux in fetal tissue, thus discouraging any appreciable exchange of sulfur back to the dam. The immensity of difference in the total ^{35}S contained in liver, kidney, heart and spleen of trimester 3-gilts and fetuses at all time periods following dose administration becomes readily apparent from data presented in table 6. Although maternal tissue ^{35}S decreased with time, maternal organs still contained as much as 10-fold that found in organs of eight fetuses after 336 hours. Labeled sulfur was detected in uterus and

TABLE 6

Total ^{35}S *in whole organs of trimester-3 gilts and fetuses at increasing time intervals after radioisotope administration* [1]

Organ	Hours after dose											
	6		24		48		120		168		336	
	Gilt	Fetus [2]	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus	Gilt	Fetus
Liver	166.1	0.58	44.2	1.94	32.2	1.46	17.6	1.24	11.3	1.03	5.8	0.76
Kidney	20.2	0.13	4.0	0.39	3.4	0.28	3.4	0.43	1.8	0.30	0.8	0.19
Spleen	14.1	0.02	7.3	0.07	4.7	0.06	3.4	0.07	2.2	0.06	1.2	0.05
Heart	18.7	0.12	2.1	0.38	2.9	0.32	1.4	0.23	1.7	0.23	0.9	0.20
Uterus and maternal placenta	405.8		133.6		146.0		127.0		56.8		47.3	
Placental fluids	5.3		49.5		21.8		66.3		21.9		41.6	
Whole fetuses [3]		22.54		144.43		99.46		151.92		138.30		131.48

[1] Expressed as percentage intravenously administered $^{35}S \times 10^{-2}$/organ, corrected to 150-kg gilts.
[2] Total quantity of ^{35}S in fetal organs standardized to eight fetuses per litter
[3] Litter size standardized to eight (1000 g) fetuses.

maternal placenta in greatest quantities after 6 hours and was observed to decrease with time. Following the rapid increase in total ^{35}S transferred to fluids and fetuses between 6 and 24 hours, little change was observed in conception product values from 24 to 336 hours.

Partition of ^{35}S by gilts. Blood and excretory data, obtained from 7-day balance studies, revealed 5.2 ± 0.2% orally administered sodium ^{35}S sulfate to be excreted in the feces and indicated that sulfate sulfur is readily and rapidly absorbed by swine. Those gilts dosed intravenously, however, retained only 24.5 ± 2.9% ^{35}S of the labeled sulfate after 168 hours. Fetuses of trimesters 1, 2 and 3 contained after 168 hours 0.01, 1.74 and 5.75%, respectively, of that ^{35}S retained by gilts. Radiochemical analyses of the total products of conception during the final trimester revealed that after 7 days 9% of that ^{35}S retained (100%) was deposited in the whole uterine complex and distributed as follows: uterus and maternal placenta (26%), placental fluids (16%) and fetuses (64%). The remaining 91% (100–9), therefore, should have been deposited in the maternal tissue. With the aid of absorption, retention and partition coefficients, calculation of maternal dietary sulfur in the form of inorganic sulfate necessary to meet fetal demands during growth and development is feasible.

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Biochemical Constituents of the Dura Mater in Vitamin A Deficiency [1]

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ABSTRACT Twenty-three male Holstein calves were fed a vitamin A-depletion ration, plus either 4, 12, 36 or 108 μg vitamin A/kg of live weight per day, for 16 consecutive weeks. Feed intake was not significantly different and growth was slightly less in those calves fed the 4 and 12 μg intakes. Cerebrospinal fluid (CSF) pressure of those animals fed the two lower intakes was elevated and inversely proportional to vitamin A intake. The tentorium cerebelli region of the dura mater, where arachnoid villi considered to be a site of CSF bulk absorption are abundant, was excised and analyzed. No significant differences were found in weight, dry matter or hydroxyproline content. Two distinct mucopolysaccharide-peptide complexes were isolated and separated on DEAE-Sephadex columns. The uronic acid content of both fractions was increased in dura tissue from the calves receiving the 4 and 12 μg intakes and inversely related to vitamin A intake. Total DNA content was not changed; however, total RNA and the ratio of RNA to DNA were greater in the tissue of those calves fed the two lower levels of vitamin A, and the magnitude of the response was inversely related to intake. It was postulated that the overproduction of the mucopolysaccharide moiety of the intercellular connective tissue matrix in the cerebral dura mater may be responsible either directly or indirectly for the increased resistance to bulk CSF absorption occurring in hypovitaminosis A.

Elevated cerebrospinal fluid (CSF) pressure in the bovine appears to be one of the first measurable physiological changes associated with vitamin A deficiency (1, 2). Recently, increased resistance to bulk CSF flow has been demonstrated in avitaminotic A-calves which also exhibited normal rates of CSF formation (3); therefore, the tissue(s) involved in the absorption process are of considerable interest. A route of CSF absorption occurs via valvular structures, arachnoid villi, situated in an intracranial connective tissue, the dura mater (4, 5). As demonstrated earlier in the dog (6), this tissue in the hypovitaminotic A-calf (7) appears to be considerably thickened. Therefore, the thickened dura might be responsible for the increased resistance to CSF flow. Because the metabolism of various connective tissue components has been postulated to be affected by vitamin A (8–10), this study was undertaken to ascertain whether some of the connective tissue constituents of the dura were affected in an uncomplicated deficiency of vitamin A.

EXPERIMENTAL

Animals and rations. Twenty-three male Holstein calves were obtained at 1 day of age from the State of Connecticut and private herds during September to December, 1967. Each animal was housed in an individual wood shaving-bedded tie stall where neither temperature nor light was controlled. Upon arrival, each calf received orally a gelatin capsule containing 34,000 μg vitamin A acetate and a 500 mg oblet of chlortetracycline,[3] the latter dose was repeated for 2 additional days. If a calf had not nursed its dam the first feeding consisted of 1.82 kg of colostrum from a frozen bank.

The experiment was divided into two periods, a growing period and a depletion-

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[3] Aureomycin, American Cyanamid Company, Princeton, N. J.

supplementation (comparison) period. During the initial growing period, 1 through 35 days of age (zero through experimental week 5), all animals received limited whole milk, limited calf starter plus chopped alfalfa hay and water ad libitum essentially as described previously (11).

Starting at 36 days of age and until the completion of the comparison period (experimental weeks 6 through 22) all animals received a vitamin A-depletion ration as described previously (11), at a rate calculated to produce a 7-day weight gain of 5%. In addition, the calves were randomly assigned, based on order of arrival, in sets of four (except the last three) to receive one of four intake levels of vitamin A: 4, 12, 36 or 108 μg retinol (fed as vitamin A acetate [4]) per kilogram live weight daily. Based on available data (12), the 4-μg and 12-μg intakes were considered, respectively, deficient and mildly deficient for maintenance of normal CSF pressure, and the 36- and 108-μg intakes, adequate. Calves designated to receive either 36 or 108 μg/kg live weight per day began supplementation on day 1 of their experimental week 6; those assigned to either 4 or 12 μg/kg live weight per day began supplementation only after the blood plasma vitamin A concentration of each animal had decreased to, or less than 5.0 μg/100 ml, for a 1-week period. In all cases, supplementation continued until day 6 of the last experimental week.

Observations. The amounts of feeds fed and refused were weighed daily to the nearest 0.05 kg (table 1).

Each animal was weighed to the nearest 0.45 kg on day 7 of each experimental week. Height at withers was taken at the end of week 5 and week 21 of the experiment. Heart rate, respiration rate and rectal temperature were taken terminally during experimental week 21.

On the last day of week 5 and at weekly intervals through week 21, blood samples (30 ml citrated) were obtained via jugular puncture for plasma vitamin A and carotenoid determination (13).

Cisternal cerebrospinal fluid pressure was measured manometrically in the unanesthetized standing position (14, 15) once during experimental week 22.

Removal of tissues. After exsanguination, the head was skinned. Two cuts were made through both the frontal bone and adhering dura mater, lateral and 3 cm on either side of the frontal suture, from the naso-frontal suture to the occipital condyles, followed by two transverse cuts along a straight line from the base of the horn to the middle of supraorbital process; and lastly, one transversely at the occipital condyles. The defined section of bone with

[4] Dry vitamin A acetate beadlets, Type 325-40, Hoffmann-LaRoche, Inc., Nutley, N. J., supplied through the generosity of Dr. R. H. Bunnell.

TABLE 1

Data for comparison period showing feed consumption and growth

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
No. of animals	6	6	6	5	—
Duration of comparison period, week	16	16	16	16	—
Feed, kg/day					
Fed	2.62	2.60	2.57	2.42	0.11
Consumed	2.55	2.47	2.54	2.37	0.14
Live weight					
Initial, kg	59.9	59.4	57.6	51.7	4.3
Gain, kg/day	0.66	0.64	0.74	0.70	0.07
Height at withers					
Initial, cm	80.0	79.6	79.5	79.4	2.0
Gain, cm/day	0.149	0.147	0.153	0.163	0.025

adherent dura mater was lifted from the remainder of the skull. The tentorium cerebelli section of the dura, where arachnoid villi are abundant, was removed by cutting the dura through the suprapineal process at the tentorium apex, then posterior and outward along the base of the tentorium to, and then transversely along, the parieto-occipital suture. After lifting the tentorium cerebelli dura from the internal plate of the parietal bone, it was rinsed twice with ice-cold 0.9% sodium chloride solution, blotted dry and stored at − 10° for 2 to 4 hours. Two separate homogenates (0.2 M sucrose and 0.05 M sodium acetate buffer, 0.005 M EDTA, 0.005 M cysteine·HCl, pH 5.5) were prepared from this tissue by dividing it bilaterally while still frozen, followed by homogenation [5] in a homogenizer [6] at 4° for 20 minutes.

A section of dura (3 cm by 3 cm) immediately anterior to the suprapineal process was taken for dry-matter determination. The adrenal glands, pituitary gland, kidneys, liver and brain were also removed at necropsy and weighed. Dura thickness was measured (table 5) to the nearest 0.01 mm with a precision thickness gauge.[7]

Determination of nitrogen, liver vitamin A and dry matter. Nitrogen content of the dura mater tissue was determined by the Kjeldahl method (16, 17) using an aliquot of the 0.25 M sucrose homogenate. Vitamin A concentration of the liver was measured by a modification of the Gallup-Hoefer procedure (18). Dry matter of dura and liver samples was determined by drying for 24 hours at 105° in a forced-draft oven.

Determination of hydroxyproline. Aliquots of the 0.25 M sucrose homogenate were hydrolyzed in 3 ml 12 N HCl with 2 ml of internal standard solution [8] or distilled water in sealed tubes at 124° for 24 hours. The hydroxyproline content of the hydrolyzate was determined spectrophotometrically [9] by the method of Prockop and Udenfriend (19).

Determination of mucopolysaccharides. Isolation of mucopolysaccharide-peptide complexes was accomplished by the method of DeLuca and Wolf (20) utilizing the total 0.05 M acetate buffer homogenate. Separation of these complexes by ion exchange-gel filtration chromatography on columns (Sephadex Type K 15/30, 1.5 cm by 30 cm) [10] of DEAE-Sephadex (Type A-25, 40–120 μ) [11] followed the procedure of Schmidt (21). Assay of the uronic acid content of the eluted fractions followed the carbazole method of Bitter and Muir (22) using glucuronolactone [12] in benzoic acid-saturated distilled water as the standard, but utilizing the reagent volumes originally suggested by Dische (23). Identification of the eluted peaks was accomplished by comparison with elution patterns of standard hyaluronic acid and chondroitin sulfate.[13] Eluted fractions of the two peaks isolated by chromatography were concentrated by lyophilization and aliquots subjected to electrophoresis [14] (250 v, 30 minutes) on cellulose-acetate strips. Visualization was accomplished by the method of DeLuca and Wolf (20). Standard hyaluronic acid [15] and chondroitin sulfate [16] were used for identification of the separated bands.

Determination of nucleic acids. DNA and RNA contents of both dura and liver tissues were measured spectrophotometrically [17] on aliquots of the respective 0.2 M sucrose homogenates following the procedure of Schneider (24), as modified for DNA by Burton (25) and for RNA by Munro and Fleck (26). Adenosine-5′-monophosphate [18] served as the orcinol reaction standard, 1.0 mg assumed to equal 1.6 mg of calf dura mater RNA and 1.8 mg of calf liver RNA (27). Deoxyadenosine-5′-monophosphoric acid [19] served as the diphenylamine reaction standard, 1.0 mg assumed to equal 2.0 mg of calf dura mater and liver DNA (27). A deoxyadenosine-5′-monophosphoric acid [20] standard was used

[5] Cousins, R. J. 1968 Biochemical constituents of the dura mater in vitamin A deficiency. Ph.D. thesis, University of Connecticut, Storrs.
[6] Virtis model 45, Virtis Company, Inc., Gardiner, N. Y.
[7] Model 25M, B. C. Ames Company, Waltham, Mass.
[8] Crystalline *dl*-hydroxyproline, Calbiochem, Los Angeles, Calif.
[9] Beckman model DU spectrophotometer, Beckman Instruments Inc., Fullerton, Calif.
[10] Pharmacia Fine Chemicals, Inc., Piscataway, N. J.
[11] See footnote 10.
[12] K and K Laboratories, Inc., Plainview, N. Y.
[13] Sigma Chemical Company, St. Louis, Mo.
[14] Microzone System, model R-101, Beckman Instruments Inc., Fullerton, Calif.
[15] See footnote 13.
[16] See footnote 13.
[17] See footnote 9.
[18] See footnote 13.
[19] See footnote 13.
[20] See footnote 13.

to correct for the contribution of DNA to the orcinol reaction.

Statistical analyses. Data were first subjected to analysis of variance (28) to estimate the variability due to vitamin A intakes (treatments) and individual animals within vitamin A intake (error). The variability due to vitamin A intakes was further divided into three orthogonal single degree of freedom contrasts as follows: 4- and 12-μg intakes versus 36- and 108-μg intakes, 4-μg intake versus 12-μg intake and 36-μg intake versus 108-μg intake. When applicable, a broken-line regression equation (29) was calculated to estimate the vitamin A intake level below which various biochemical responses deviated in a linear manner from "normal" levels.

RESULTS

Animals. Actual age at the beginning of the comparison period, feed fed and consumed and growth are presented in table 1. Due to the method of feeding based on initial live weight, the amount fed was related to the initial weight of each group of calves. This influenced the amount of feed actually consumed, but the differences in feed consumed were not statistically significant. When feed consumed was expressed as a percentage of that fed (percentages transformed to arcsin $\sqrt{\%}$), there were no statistically significant differences at $P \leq 0.05$. Live weight increase was slightly less ($P \leq 0.05$) in those calves receiving the 4- and 12-μg intakes. Height at withers followed this same trend, but was without statistical significance.

Observations and general analyses. Average terminal heart rate equaled 104 beats/minute with a SD of 14, respiration rate 24 cycles/minute with a SD of 7 and rectal temperature 39.08° and a SD of 0.22. No statistically significant differences among groups were found for any of these criteria. Cisternal cerebrospinal fluid pressure (table 2), as expected, was substantially higher in those calves receiving the lower vitamin A intakes. The common logarithm of CSF pressure, Y_1, was related to the log vitamin A intake in microgram per kilogram live weight per day, X, as follows:

$$Y_1 = 1.76 - 0.6588\,(X - 1.682)\theta, \text{ SD} \pm 0.17, \text{ with } \theta = 0 \text{ when } X > 1.682 \text{ and } \theta = 1 \text{ when } X \leq 1.682$$

Therefore, in this study cisternal CSF pressure was elevated at an estimated vitamin A intake of less than 48 μg/kg live weight per day. In evaluating these statistics, it is necessary to note that cisternal CSF pressure was successfully measured in only three of the six calves fed the lowest intake of vitamin A. As expected (30), high incidence of papilledema and convulsions were evident in the calves receiving the lowest intake of 4 μg (table 2).

Vitamin A concentrations in both plasma and liver are reported in table 3. Plasma concentrations increased with the common logarithm of vitamin A intake, and when the lowest intake of 4 μg was not included,

TABLE 2

Effect of vitamin A intake upon cisternal cerebrospinal fluid pressure and incidence of convulsions and papilledema

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
Cisternal cerebrospinal fluid pressure, mm of saline					
Actual	241 [1]	180	63 [2]	68 [3]	—
Log_{10}	2.38 [1]	2.25	1.78 [2]	1.76 [3]	0.17
No. of calves exhibiting convulsions	4	1	0	0	—
No. of calves exhibiting papilledema	6	2	0	0	—

[1] Based on three calves instead of six.
[2] Based on four calves instead of six.
[3] Based on four calves instead of five.

TABLE 3

Effect of vitamin A intake upon plasma and liver vitamin A concentration

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
Plasma vitamin A, μg/100 ml					
Initial	13.0	14.6	12.2	10.6	3.6
Avg	4.2	8.6	19.3	32.3	1.9
Terminal	2.4	7.8	18.1	32.0	2.2
Liver vitamin A, μg/100 g					
Actual	2	47	932	6092	—
Log_{10}	0.03	1.63	2.95	3.78	0.34

TABLE 4

Effect of vitamin A intake upon the weight of brain, kidneys and pituitary gland

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
Brain					
Total, g	315	338	343	346	22
Per unit live wt, g/kg	2.45	2.55	2.40	2.56	0.31
Kidney					
Left, g	298	263	246	226	41
Right, g	290	256	241	228	42
Total, g	588	519	487	454	80
Per unit live wt, g/kg	4.6	3.9	3.4	3.4	1.0
Pituitary, g	1.96	1.54	1.43	1.44	0.28

it increased in essentially a linear fashion. In the case of liver, the common logarithms of the vitamin A concentration followed essentially the same trends with the common logarithm of vitamin A intake as did plasma.

The left and right adrenal glands of the average calf weighed 7.8 g with a SD of 1.2, and the liver weighed 2365 g with a SD of 206. The kidneys and pituitary gland weights (table 4) were heavier in the group receiving the 4-μg intake of vitamin A. The brains in the lowest vitamin A-intake group were smaller, however, when expressed on a gram per kilogram of live weight basis; this difference was not observed. In four of the six calves of the 4-μg intake group pituitary cysts were observed, a condition reported previously (30), and were responsible for the greater average weight exhibited by this group.

Biochemical analyses of dura mater. Thickness measurements (table 5) reflected a dural thickening in the animals receiving the lowest vitamin A intake. Measurement no. 5 was suggestive of a progressive thickening process. The change in measurement no. 5, Y_2, was related to the log vitamin A intake in micrograms per kilogram live weight per day, X, as follows:

$$Y_2 = 0.33 - 0.2485\ (X - 1.442)\theta;\ SD \pm 0.18,$$
with $\theta = 0$ when $X > 1.442$ and $\theta = 1$ when $X \leq 1.442$

The thickness of the dura at measurement no. 5 occurred at an estimated vitamin A intake of less than 28 μg/kg live weight per day. Sample weights of the excised tentorium cerebelli dural region averaged 6.4 g with a SD of 1.4. Only the dura tissue from the severely deficient animals fed the 4-μg intake had a slightly lower dry matter content.

Hydroxyproline concentration (milligrams per gram fresh tissue) appeared to be slightly greater in the dura tissue of the calves fed the 4-μg intake (table 5), but this difference was not of sufficient magnitude to be statistically significant. Express-

TABLE 5

Effect of vitamin A intake upon thickness, dry matter plus hydroxyproline and nitrogen concentrations of the dura mater

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
Thickness, mm					
Measurement no. 1 [1]	1.22	0.54	0.44	0.66	0.32
Measurement no. 2 [2]	1.46	1.00	1.05	0.82	0.41
Measurement no. 3 [3]	0.69	0.43	0.36	0.41	0.20
Measurement no. 4 [4]	0.78	0.62	0.76	0.58	0.16
Measurement no. 5 [5]	0.54	0.42	0.34	0.33	0.18
Measurement no. 6 [6]	0.82	0.32	0.42	0.32	0.13
Dry matter, g/100 g	20.2 [7]	22.9	22.2	21.8 [8]	1.9
Hydroxyproline, mg/g	17.0	16.1	14.9	15.9	2.2
Nitrogen, mg/g	28.3	28.7	27.4	28.6	1.9

[1] Falx cerebelli, 5 cm anterior to the parieto-frontal suture on midline.
[2] Falx cerebelli, 6 cm anterior to the parieto-frontal suture on midline.
[3] Frontal bone dura, 1.5 cm anterior to the parieto-frontal suture and 1.0 cm lateral of midline.
[4] Basi-occipital bone dura, 1.0 cm anterior to posterior margin of basi-occipital bone and lateral to the bulky subdural venous system.
[5] Dura on 1st cervical vertebra, middorsally.
[6] Dura on sphenoid bone, 5 mm anterior to midline of optic foramina.
[7] Based on five calves instead of six.
[8] Based on four calves instead of five.

ing concentration on a dry matter basis accentuated this difference.

The concentrations (as micrograms uronic acid per gram fresh tissue) of total isolated mucopolysaccharides (peak I + peak II), as well as that of each separated fraction, peak I (hyaluronic acid) and peak II (chondroitin sulfate), were higher in those animals fed the 4- and 12-μg intakes of vitamin A (fig. 1). These differences in the uronic acid content of peak I, Y_3; peak II, Y_4; and the total (peaks I + II), Y_5; were related to the log vitamin A intake in micrograms per kilogram live weight per day, X, as follows:

$Y_3 = 43\text{–}31.9573\ (X\text{–}1.422)\theta$, SD $\pm$ 13, with $\theta = 0$ when $X > 1.422$ and $\theta = 1$ when $X \leq 1.422$

$Y_4 = 272\text{–}463.0879\ (X\text{–}1.222)\theta$, SD $\pm$ 63 with $\theta = 0$ when $X > 1.222$ and $\theta = 1$ when $X \leq 1.222$

$Y_5 = 316\text{–}498.2014\ (X\text{–}1.232)\theta$, SD $\pm$ 68 with $\theta = 0$ when $X > 1.232$ and $\theta = 1$ when $X \leq 1.232$

The changes in uronic acid content of peak I, peak II and total (peaks I + II) were affected at an estimated vitamin A intake of less than 26, 17 and 17 μg/kg live weight per day, respectively. When the uronic acid concentrations were calculated on a dry-matter basis, the same general trends were apparent.

Electrophoretic separation of the individual mucopolysaccharide-peptide complexes, obtained from the dura mater of one calf fed the 4-μg intake and one calf fed the 108-μg intake, appeared as individual bands. Peak I and peak II moved 2.2 cm and 3.5 cm, respectively, whereas standard hyaluronic acid and chondroitin sulfate moved 2.1 cm and 3.5 cm, respectively.

The concentration of deoxyribonucleic acid (DNA, milligrams per gram fresh tissue) was not significantly affected by vitamin A intake (fig. 2), whereas both ribonucleic acid (RNA, milligrams per gram fresh tissue) and the ratio of RNA to DNA were found to be elevated in the dural tissue of those calves receiving the two lower intakes of vitamin A. The differences in RNA, Y_6; and RNA/DNA, Y_7; were related to the log of vitamin A intake in micrograms per kilogram live weight per day, X, as follows:

$Y_6 = 1.14\text{–}0.2557\ (X\text{–}1.552)\theta$, SD $\pm$ 0.28, with $\theta = 0$ when $X > 1.552$ and $\theta = 1$ when $X \leq 1.552$

$Y_7 = 1.78\text{–}0.3347\ (X\text{–}1.712)\theta$, SD $\pm$ 0.23, with $\theta = 0$ when $X > 1.712$ and $\theta = 1$ when $X \leq 1.712$

The elevation in RNA content occurred at an estimated vitamin A intake of less than

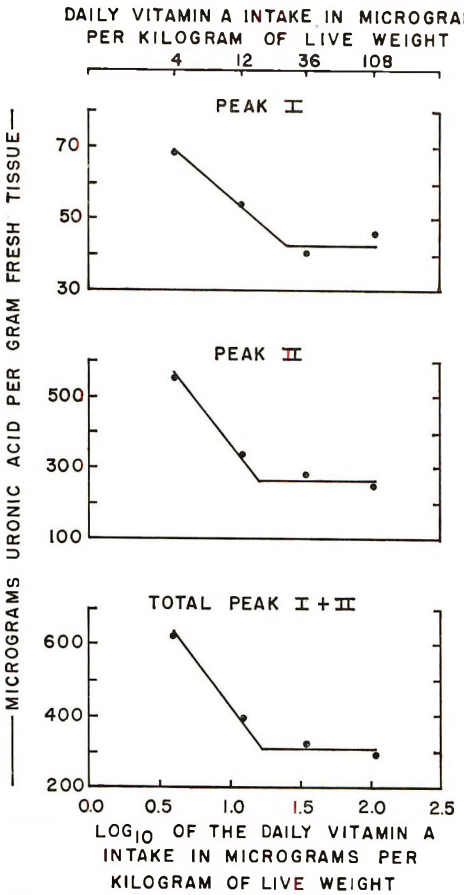

Fig. 1 Regressions of uronic acid content of mucopolysaccharide-peptide complexes isolated from the dura mater on vitamin A intake.

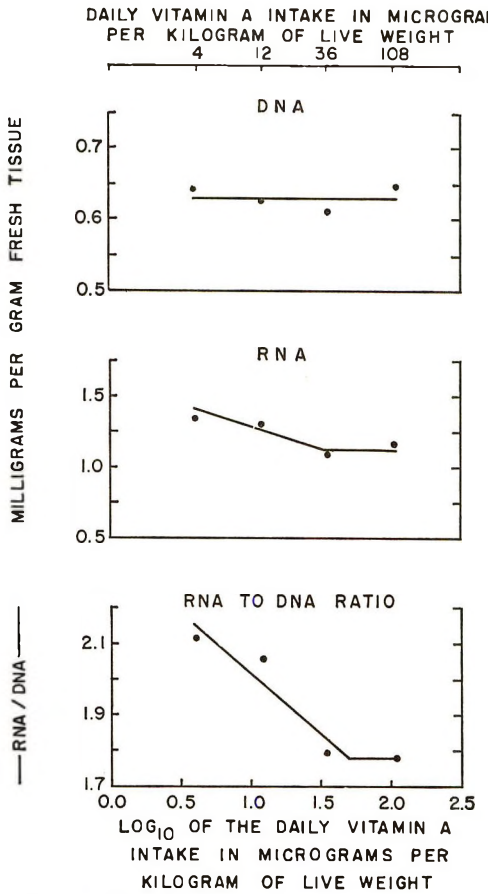

Fig. 2 Regressions on DNA and RNA content plus the RNA-to-DNA ratio of the dura mater on vitamin A intake.

36 μg/kg live weight per day; the increase in RNA/DNA occurred at an estimated vitamin A intake of less than 52 μg/kg live weight per day.

No differences in dura mater total nitrogen content (milligrams per gram fresh tissue) were observed among the treatment groups (table 5).

When the concentration of DNA was calculated on a dry-matter basis, the dura mater from the most deficient calves, 4-μg intake, contained greater DNA, but this was not of sufficient magnitude to be of statistical significance at $P \leq 0.05$. When RNA was expressed on a dry-matter basis, the same general trend as found for the

TABLE 6

Effect of vitamin A intake upon the dry matter and nucleic acid content of the liver

	Vitamin A intake, μg/kg live wt per day				SD per calf
	4	12	36	108	
Dry matter, g/100 g	25.4	27.5	27.6	27.4	1.6
Ribonucleic acid, mg/g	5.62	5.38	5.38	5.33	0.37
Deoxyribonucleic acid, mg/g	3.40	3.07	2.95	3.02	0.42
Ribonucleic acid-to-deoxyribonucleic acid ratio	1.70	1.76	1.82	1.79	0.20

fresh tissue was present. Dura mater nitrogen content per unit of dry matter was elevated in the tissues of the calves fed the lowest vitamin A intake.

Biochemical analyses of liver. Liver dry matter of those calves fed the 4-μg intake was slightly less; RNA (milligrams per gram fresh tissue) slightly greater; DNA (milligrams per gram fresh tissue) slightly greater; and the ratio of RNA to DNA slightly less (table 6). Only the differences in the dry matter and RNA content per unit dry matter between the 4- and 12-μg intake groups were significant at $P \leq 0.05$.

DISCUSSION

The results on dura mater composition (table 5 and figs. 1 and 2) provide evidence that metabolic changes occur in dura mater connective tissue during vitamin A deficiency. The collagen component of the intercellular matrix, as measured by hydroxyproline content, was found not to be significantly changed. This is in agreement with Havivi and Wolf (10) who were unable to find a difference in epiphyseal bone-collagen hydroxyproline concentration in the vitamin A-deficient chick. Moreover, since it has been shown that peptide-bound proline hydroxylation necessary for collagen formation is ascorbic acid-dependent (31), the present data and those previously obtained on whole blood and plasma ascorbic acid concentrations in vitamin A-deficient calves (32) indirectly support the recent suggestion of Zile and DeLuca (33) that ascorbic acid biosynthesis is not affected by vitamin A deficiency.

In contrast to hydroxyproline content of the dura mater, there was a marked increase in mucopolysaccharide concentration of this tissue in the vitamin A-deficient calves fed the 4- and 12-μg vitamin A intakes, and the increase was related to the extent of the deficiency. Since this increase occurred in both isolated mucopolysaccharide-peptide fractions, which were tentatively identified as hyaluronic acid (peak I) and chondroitin sulfate (peak II) (fig. 1), it appears that the degree of enzymatic sulfation of polysaccharide precursors is probably not altered in the deficient animals, although this cannot be directly established from these data. The estimates establishing the intake level below which this increase occurs support the hypothesis that this change occurs relatively early in vitamin A deficiency; and that the magnitude of the change parallels the degree of the deficiency.

The above results are in agreement with those of Frape et al. (34) who reported increased $^{35}SO_4^=$ uptake into bone, cartilage and lung tissue in avitaminotic A pigs; of DeLuca and Wolf (20) who found an increase in mucopolysaccharide content of lung tissue from pair-fed deficient rats; and of Havivi and Wolf (10) who reported a higher uronic acid content of epiphyseal bone matrix from deficient chicks. The latter report is of interest, since cerebral dura mater serves as the periosteum for at least a portion of the frontal bone (35). Hence, the excessive mucopolysaccharide content observed in the present study could well be the first step in altered cranial bone formation observed in avitaminotic A-calves (7).

The agreement of the present study with the above reports provides fairly firm biochemical evidence for a tissue change involving the mucopolysaccharide moiety. More latitude is necessary, however, to explain the elevated CSF pressure in relation to this tissue change. The model for connective tissue structure proposed by Mathews (36) is compatible with an accelerated rate of mucopolysaccharide synthesis. Mucopolysaccharide side chains extending from a central mucoprotein core impart a high degree of interaction with collagen fibrils and are responsible for maintaining intercellular matrix stability. It is easy to visualize how changes in the mucopolysaccharide moiety could drastically alter the structure, and hence, the properties of this highly ordered tissue matrix. Since arachnoid villi which are responsible for considerable CSF bulk absorption are embedded within the dura mater (4, 5), a modification of the macromolecular structure of the dura could indirectly alter the physiological function of these villi, which could in turn be responsible for the elevated CSF pressure observed in avitaminosis A.

A second possible explanation of the impaired CSF bulk absorption would involve the structural integrity of arachnoid villi. Since the arachnoid granulations, aggrega-

tions of individual villi, are believed rich in mucopolysaccharides (37), any change in these macromolecules could directly affect the integrity of the villi and thereby result in decreased functional ability. It needs to be noted here that Wolbach (38) reported herniations of the cerebellum and cerebrum into dural sinuses at sites of arachnoid villi, and attributed this to faulty connective tissue or bone growth. Lastly, the cells lining these structures, particularly those of the tubules (5), could also be modified and result in reduced clearance of CSF into the venous system.

The DNA content of the dura was very low as expected since all connective tissues are characteristically hypocellular. Moreover, the concentrations of DNA were not significantly different among vitamin A-intake levels employed in this study, providing evidence that the actual cell content was not affected by deficient intakes of the vitamin. In contrast to DNA, there was a progressive increase in RNA concentration with decreasing vitamin A intakes below an estimated intake of 36 μg, and consequently the RNA-to-DNA ratio (fig. 2) also increased. Since none of these criteria, when expressed on a fresh tissue basis, were significantly altered in the liver (table 6), some specificity of action of vitamin A on RNA synthesis in the dura may be present.

Though the response of RNA content may involve all major types of RNA, selective effects could not be detected by the methods employed, and would necessitate a different type of experiment. It is of interest that in 12 hypervitaminotic A calves, the ratio of RNA to DNA in dural tissue was less than in 12 control calves.[21] Although the significance of the nucleic acid change found in the present study is not known at present, one is reminded of the postulated regulatory function for vitamin A proposed by Olson (39).

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Influence of Ethionine on Choline-deficiency Fatty Liver [1]

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ABSTRACT Rats fed ad libitum a choline-deficient diet containing 0.25 or 0.5% ethionine for 7 to 21 days did not develop a choline-deficiency fatty liver. Likewise, rats force-fed a controlled intake of choline-deficient diet either supplemented with ethionine or receiving ethionine intraperitoneally for 3 days did not develop fatty liver. Rats force-fed the choline-supplemented or choline-deficient diet containing ethionine developed a small increase in liver lipid after 1 day, particularly if prefasted for 1 day, which diminished after 2 or 3 days. After induction of choline-deficiency fatty liver, the addition of ethionine to the deficient diet slowly decreased the lipid accumulation. The addition of methionine to the choline-deficient, ethionine-supplemented diet partially counteracted the inhibitory effect of ethionine on choline-deficiency fatty liver. Thus, ethionine, a compound which itself induces fatty liver when administered to fasted animals, is capable of inhibiting the induction of fatty liver due to choline deficiency.

The rapid induction of fatty liver in the rat due to choline deficiency and due to administration of ethionine has been reported from many laboratories (1–3), and concepts concerning the pathogenesis of fatty liver due to each in acute experiments have been reviewed (3–5). In long-term experiments both ethionine supplementation to the diet (6) and choline deficiency (1, 7) have been found to induce liver cancers. In the case of the latter, however, the possibility that aflatoxin contamination of the peanut meal used may have played a role must be considered (8).

During the course of a study concerned with the influence of fatty liver on the induction of liver tumorigenesis due to chemical hepatocarcinogens, an interesting observation relating to the effect of ethionine on the induction of fatty liver by feeding a choline-deficient diet was encountered. The addition of 0.25 or 0.5% DL-ethionine to a choline-deficient diet inhibited the induction of fatty liver in rats fed for periods up to 21 days. A similar result had been reported earlier by Hardwick and Winzler (9) in young rats fed ad libitum a low methionine, low choline diet for 12 days. This study reports the observations of the effect of ethionine on the induction of fatty liver in young rats fed ad libitum or force-fed a choline-deficient diet.

MATERIALS AND METHODS

Male and female rats of the Sprague-Dawley strain [2] were used. The animals were fed a commercial ration [3] and weighed between 60 g and 130 g at the start of the experiments. In all experiments several groups of rats, each of the same sex, age and weight were used.

The choline-deficient (CD) and choline-supplemented (CS) diets were similar to those of Young et al. (10). The vitamin mix (8) used contributed the following in milligrams per gram diet mixture: thiamine·HCl, 0.2; riboflavin, 0.075; pyridoxine·HCl, 0.2; calcium pantothenate, 0.52; nicotinic acid, 0.75; folic acid, 0.02; biotin, 0.004; 2-methyl-1,4-naphthoquinone, 0.1; cyanocobalamin (vitamin B_{12}), 0.002; and oleum percomorphum, 0.02 ml. In some experiments (those described in tables 3 to 6) the vitamin mix was increased fivefold. This increase in vitamins did not influence our experimental results. The CS diet contained either 0.6 or 0.8% choline chloride. Supplements, when added to the diets, consisted of 0.25 or 0.5% DL-ethio-

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[2] Sprague-Dawley, Madison, Wis.
[3] Wayne Lab-Blox, Allied Mills Inc., Chicago, Ill.

nine, 0.25% L-methionine or 0.25% adenine sulfate.

In ad libitum experiments the diets were offered as dry mixtures. Diet consumption was measured by weighing diet jars at regular intervals. In force-feeding experiments the rations were blended with distilled water so that each milliliter contained 0.5 g diet. The rats were force-fed three times daily according to the method of Shay and Gruenstein (11). The animals received an average daily feeding of 1 g ration/10 g initial body weight. All rats had free access to water.

Rats were housed in individual wire cages with raised bottoms and kept in an air-conditioned room maintained at 25.5°. Rats were weighed at the beginning and end of each experiment. In addition, in the ad libitum experiments (table 1) the animals were weighed at 3-day intervals. The animals were killed in the morning by decapitation. The livers were weighed fresh. The methods used for chemical analyses have been described in detail in earlier studies (12–14). Radioactivity in protein was measured using a liquid scintillation spectrometer.[4] The level of free methionine of pooled livers of control and experimental animals was determined in an amino acid analyzer,[5] and its radioactivity was counted in a liquid scintillation spectrometer [6] (at approximately 50% counting efficiency), through which the separated amino acid passed before returning to the amino acid analyzer for quantitative determination.

RESULTS

In four experiments, young rats were fed ad libitum a choline-deficient (CD) or a choline-supplemented (CS) diet with or without 0.25 or 0.5% DL-ethionine for 3, 7, 14 or 21 days. Table 1 summarizes the diet consumption, body weight changes and hepatic weight, lipid, protein and choline oxidase activity of the different groups of animals. Animals fed the CD or CS diet for 3, 7 or 14 days consumed about the same amounts of diet each day, but animals fed the deficient diet gained somewhat less weight. By day 21 rats fed the CD diet consumed less (23%) diet and gained less (64%) weight than comparable animals fed the CS diet. In contrast, when ethionine was supplemented to the CD or CS diet, the animals consumed much less (49 to 87%) diet and lost weight in the 7, 14 or 21 day experiments.

Liver weights were increased in animals fed the CD diet in comparison with those fed the CS diet. Addition of ethionine to the CS diet caused either no change or a decrease in liver weights; addition of ethionine to the CD diet caused a marked decrease in liver weights. Hepatic total lipids were significantly elevated in rats fed the CD diet for 3, 7, 14 or 21 days. Such elevations, however, did not develop when ethionine was added to the CD or CS diet. Liver protein was markedly decreased in rats fed the CD or CS diet supplemented with ethionine. Hepatic choline oxidase activity decreased when rats were fed the CD diet. Ethionine addition decreased hepatic choline oxidase activity in rats fed the CS or CD diet.

From the results in table 1, as well as from earlier data by Hardwick and Winzler (9), it was impossible to decide whether the inhibitory action of ethionine was due to a primary effect of ethionine or due to a secondary effect related to suppressed diet intake and the loss of body weight. For this reason a second series of experiments was conducted in which the diets were force-fed to ensure a constant, controlled intake rather than the unequal intake encountered with ad libitum feeding (table 1).

Groups of rats were tube-fed the CS or CD diet with or without 0.25 or 0.5% ethionine for 3 days. During this interval all animals received daily on the average 1 g diet/10 g initial body weight. The results of body weight changes and hepatic weight, lipid, protein and choline oxidase activity are summarized in table 2. Animals force-fed the CS or CD diet gained approximately the same amounts of weight and had similar values for liver weight and hepatic protein. Rats fed the CD diet, however, had a significant increase in hepatic lipid and a decrease in choline oxidase activity. These results are similar to but less marked than those found in

[4] Packard Tri-Carb, Packard Instrument Company, Inc., Downers Grove, Ill.
[5] Spinco Model 120B, Beckman Instruments, Inc., Palo Alto, Calif.
[6] Nuclear-Chicago Corporation, Des Plaines, Ill.

TABLE 1

Body weight changes and hepatic weight, lipid, protein and choline oxidase activity of rats fed ad libitum a choline-deficient or choline-supplemented diet with or without ethionine

Exp. no.	Duration	Group [1]	Sex	No. of rats	Diet intake	Onset wt	Wt at death	Wt change	Liver						
									Wt	Lipid		Protein		Choline oxidase	
	days				*g/day*	*g*	*g*	*g*	*g/100 g body wt*	*%*	*mg/liver/ 100 g body wt*	*%*	*mg/liver/ 100 g body wt*	*units/g liver*	*units/liver/ 100 g body wt*
136	3	CS	F	3	6.7	68	73	+ 5	4.65 ± 0.27 [2]	5.4 ± 0.9 [2]	247 ± 31 [2]	18.5 ± 1.0 [2]	862 ± 41 [2]	870 ± 14.2 [2]	4047 ± 149 [2]
		CD	F	3	7.8	69	73	+ 4	5.50 ± 0.26	15.0 ± 1.2 [3]	804 ± 20 [3]	18.9 ± 0.2	1041 ± 56	579 ± 110.7	3179 ± 620
137	7	CS	F	3	14.7	115	135	+20	5.23 ± 0.21	4.5 ± 0.4	236 ± 3	14.4 ± 0.4	751 ± 36	(1) [4] 869	4970
		CD	F	3	12.9	116	131	+15	5.67 ± 0.27	21.9 ± 2.5 [3]	1239 ± 126 [3]	13.8 ± 1.8	672 ± 47	(2) 700 ± 10.0	4084 ± 173
124	7	CS	F	2	10.2	94	115	+21	4.98 ± 0.16	4.5 ± 0.3	219 ± 2				
		CD	F	2	9.7	89	107	+18	5.42 ± 0.23	26.6 ± 1.0 [3]	1442 ± 107 [3]				
		CS + E [5]	F	2	1.6	88	63	−25	4.03 ± 0.28	4.8 ± 0.5	192 ± 7				
		CD + E [5]	F	2	1.3	94	64	−30	2.69 ± 0.08 [3]	6.6 ± 0.2 [6]	177 ± 2 [3]				
	14	CS	F	2	10.3	86	117	+31	4.47 ± 0.17	5.0 ± 0.1	223 ± 4				
		CD	F	2	10.2	93	118	+25	7.44 ± 0.19 [3]	36.9 ± 10.3	2762 ± 832				
		CS + E [5]	F	2	3.2	87	57	−30	4.49 ± 0.11	4.5 ± 0.4	200 ± 15				
		CD + E [5]	F	2	3.0	92	49	−43	3.27 ± 0.11 [6]	4.7 ± 0.6	152 ± 23				
132	21	CS	M	4	10.9	110	165	+55	4.86 ± 0.21	4.3 ± 0.1	208 ± 9	17.4 ± 0.3	847 ± 36	1510 ± 117	7302 ± 420
		CD	M	4	8.4	112	132	+20	7.77 ± 0.26 [3]	37.9 ± 1.4 [3]	3105 ± 226 [3]	17.0 ± 1.4	1304 ± 120 [6]	487 ± 76 [3]	3748 ± 609 [3]
		CS + E [7]	M	4	4.3	110	85	−25	4.07 ± 0.22 [6]	4.4 ± 0.3	177 ± 8 [6]	15.7 ± 0.3	639 ± 34 [3]	291 ± 9 [3]	1190 ± 89 [3]
		CD + E [7]	M	4	3.5	109	74	−35	4.30 ± 0.24	4.4 ± 0.3	190 ± 13	16.2 ± 0.5	694 ± 46 [6] (3)	340 ± 180 [3]	1298 ± 1112 [3]

[1] CS = choline-supplemented diet; CD = choline-deficient diet; E = ethionine supplement.
[2] Mean ± SE. All comparisons are made with CS group of each experiment.
[3] $P < 0.01$.
[4] Number of determinations in parentheses if different than number of animals.
[5] 0.5% DL-ethionine.
[6] $0.05 > P > 0.01$.
[7] 0.25% DL-ethionine.

TABLE 2

Body weight changes and hepatic weight, lipid, protein and choline oxidase activity of rats force-fed a choline-deficient or choline-supplemented diet with or without ethionine for 3 days

Group [1]	No. of rats	Onset wt	Wt at death	Wt change	Liver								
					Wt	Lipid			Protein			Choline oxidase	
		g	*g*	*g*	*g/100 g body wt*		%	*mg/liver/100 g body wt*	%	*mg/liver/100 g body wt*		*units/g liver*	*units/liver/ 100 g body wt*
CS	24	94	104	+10	4.11 ± 0.08 [2]	(23)[3]	4.5 ± 0.1 [2]	179 ± 5.1 [2]	(17)[3]	18.3 ± 0.8 [2]	755 ± 18.1 [2]	(12)[3]	1358 ± 136 [2]	5452 ± 454 [2]
CD	22	95	103	+ 8	4.15 ± 0.11	(20)	9.4 ± 0.5 [5]	388 ± 28.7 [5]	(16)	17.5 ± 1.3	734 ± 32.0	(15)	1034 ± 97	4199 ± 318 [6]
CS + E [4]	25	93	98	+ 5	3.67 ± 0.04 [5]	(24)	5.5 ± 0.2 [5]	198 ± 5.3	(19)	18.0 ± 0.4	654 ± 30.7 [5]	(17)	1010 ± 83 [6]	3746 ± 319 [5]
CD + E [4]	24	100	105	+ 5	3.30 ± 0.06 [5]	(23)	5.6 ± 0.4 [6]	184 ± 13.3	(16)	17.9 ± 0.7	588 ± 49.1 [5]	(14)	1058 ± 116	3638 ± 382 [5]
CS + E [7]	5	89	94	+ 5	3.63 ± 0.08 [5]	(4)	6.1 ± 0.6 [6]	225 ± 23.0	(3)	15.4 ± 0.7 [6]	568 ± 27.8 [5]	(2)	1003 ± 91 [6]	3608 ± 111 [5]
CD + E [7]	3	90	96	+ 6	3.68 ± 0.17 [6]	(3)	5.5 ± 0.3 [5]	201 ± 18.7	(3)	15.2 ± 0.7 [5]	561 ± 12.0 [5]	(3)	750 ± 112 [5]	2735 ± 342 [5]
CS + E [8]	2	79	82	+ 3	3.19 ± 0.18 [5]		6.0 ± 0.9	184 ± 19.0						
CD + E [8]	4	74	74	0	3.06 ± 0.15 [5]		5.6 ± 0.1 [5]	171 ± 10.9						

[1] CS = choline-supplemented diet; CD = choline-deficient diet; E = ethionine.
[2] Mean ± SE. All comparisons were made with CS group.
[3] Number of determinations in parentheses if different than number of animals.
[4] 0.25% DL-ethionine.
[5] $P < 0.01$.
[6] $0.05 > P > 0.01$.
[7] DL-ethionine (22.5 mg/rat/day) administered intraperitoneally twice daily.
[8] 0.5% DL-ethionine.

rats fed the same diets ad libitum, particularly in regard to hepatic lipid accumulation (table 1). When 0.25 or 0.5% ethionine was added to the CS or CD diet, the animals gained less weight; the hepatic weights, protein content and choline oxidase activity decreased; and the liver lipid level was not increased, when compared with comparable groups of animals. The latter observation indicated that ethionine suppressed the accumulation of hepatic lipids due to choline deficiency even when the diet intake was carefully controlled.

To determine whether the action of ethionine might reside within the gastrointestinal tract, possibly influencing the metabolism of the bacterial flora or by altering intestinal absorption of dietary components, an experiment was performed in which ethionine was administered intraperitoneally rather than orally by stomach tube with the diet mixture. The results (table 2) indicate that ethionine inhibits the increase in hepatic lipids due to the CD diet even when it is administered intraperitoneally.

It was apparent from a comparison of the results in tables 1 and 2 that rats fed the CD diet ad libitum for 3 days developed a more marked degree of fatty liver than did the animals force-fed the same diet. In an attempt to gain more insight into the factor or factors responsible for this difference, the following series of experiments were conducted. Because in the force-feeding experiments the animals were tube-fed the CS diet for 1 or 2 days before being tube-fed the CD diet, it was thought that this prefeeding might build up a reserve level of choline in the liver and thereby delay the development of the deficient state. Therefore, in two experiments (exps. A and B, table 3) rats were either fasted or tube-fed the CS diet for 1 day and then tube-fed the CD diet. The results indicated that after 1 day a greater accumulation of liver lipid due to the CD diet developed in the prefasted group (+ 90%) than in the pre-CS diet-fed group (+ 23%). As the experimental period was extended to 2 or 3 days after the prefeeding (exp. B, table 3), the liver lipid increased appreciably (45 and 66%, respectively) in the CD group over the comparable controls. Supplementation of ethionine to the CS or CD diet for 1 day after prefeeding, or particularly after fasting, led to an increase in liver lipid which then decreased after 2 or 3 days. In a third experiment (exp. C, table 3) rats were fasted for 1 day and then fed ad libitum the CS or CD diet with or without ethionine for 1 day. There was a marked increase in liver lipid in the CD group and moderate increases in the groups fed CS plus ethionine or CD plus ethionine. Again, there was an early rise in liver lipid in the CS group due to ethionine. In a fourth experiment (exp. D, table 3) rats were fasted 24 hours and then divided into three major groups; one group was fed 8 hours ad libitum and killed; another group was fed 8 hours ad libitum and killed 16 hours later; the final group was fed 24 hours ad libitum and then killed. Within each group subgroups of rats were fed ad libitum the CS or CD diet with or without ethionine. In group D–1 (animals fed the different diets for 8 hours) no lipid accumulation occurred. In group D–2 (fed 8 hours and killed after 24 hours) rats fed the CD diet had a 38% increase in liver lipid, rats fed the CS diet plus ethionine had a 86% increase, and rats fed the CD diet plus ethionine had a 100% increase. In group D–3 (fed for 24 hours) all three experimental subgroups had a 58% increase in liver lipid over that of the CS group. The results of the experiments summarized in table 3 indicated that the addition of ethionine to the CS diet caused a rise in liver lipid during the first 24 hours. After 2 to 3 days, however, this transient rise in liver lipid disappeared and the liver lipid accumulation due to choline deficiency was inhibited.

In an attempt to determine whether ethionine would have any influence on the lipid accumulation after the choline-deficient fatty liver has already developed, three experiments were performed. In experiment 1 (table 4) rats were fed ad libitum the CD diet for 3 days by which time they developed a fatty liver and then were divided into four groups according to diet, CD or CS with or without 0.25% ethionine. Rats were then force-fed the four diets for 3 days. The results indicate that rats force-fed the CD diet retained a marked elevation in hepatic lipids. This was moderately decreased (42%) in the

TABLE 3

Influence of fasting or prefeeding on liver lipid accumulation in rats fed a choline-supplemented or choline-deficient diet with or without ethionine

Exp. no.	Experimental conditions [1]						No. of rats	Liver lipid	
	Pretreatment			Treatment					
	Diet	Route	Duration	Diet	Route	Duration			
			hours			*hours*		%	*mg/liver/100 g body wt*
A	Fast	—	24	CS	FF	24	4	3.8 ± 0.3 [2]	152 ± 10.9 [2]
	Fast		24	CD	FF	24	4	6.8 ± 0.1 [3]	288 ± 6.8 [3]
	Fast		24	CS+E	FF	24	4	7.6 ± 1.0 [4]	342 ± 40.4 [3]
	Fast		24	CD+E	FF	24	4	8.0 ± 0.5 [3]	362 ± 26.0 [3]
B	CS	FF	24	CS	FF	24	2	4.3 ± 0.3	197 ± 14.3
	CS	FF	24	CD	FF	24	2	5.8 ± 0.6	242 ± 0
	CS	FF	24	CS+E	FF	24	2	5.9 ± 0.7	284 ± 18.5
	CS	FF	24	CD+E	FF	24	2	6.0 ± 0.1 [4]	260 ± 20.0
	CS	FF	24	CS	FF	48	2	4.4 ± 0.1	193 ± 13.0
	CS	FF	24	CD	FF	48	2	6.5 ± 0.3 [4]	280 ± 50.0
	CS	FF	24	CS+E	FF	48	1	5.2	205
	CS	FF	24	CD+E	FF	48	3	6.0 ± 0.4 [4]	241 ± 19.0
	CS	FF	24	CS	FF	72	2	4.7 ± 0.2	211 ± 17.0
	CS	FF	24	CD	FF	72	2	7.7 ± 0.5 [4]	351 ± 2.0 [4]
	CS	FF	24	CS+E	FF	72	2	6.5 ± 1.3	247 ± 49.0
	CS	FF	24	CD+E	FF	72	2	5.6 ± 0.1	207 ± 18.0
C	Fast	—	24	CS	AL	24	3	4.6 ± 0.5	234 ± 12.4
	Fast		24	CD	AL	24	3	8.2 ± 0.6 [4]	439 ± 31.1 [3]
	Fast		24	CS+E	AL	24	3	8.0 ± 0.1 [3]	365 ± 24.1 [3]
	Fast		24	CD+E	AL	24	3	9.0 ± 0.6 [3]	393 ± 37.9 [4]
D-1	Fast	—	24	CS	AL	8	2	3.6 ± 0	189 ± 6.6
	Fast		24	CD	AL	8	3	4.1 ± 0.6	188 ± 21.2
	Fast		24	CS+E	AL	8	2	3.5 ± 0.1	171 ± 15.1
	Fast		24	CD+E	AL	8	3	3.7 ± 0.4	163 ± 8.7
D-2	Fast		24	CS	AL	8–24 [5]	2	3.8 ± 0.6	170 ± 11.7
	Fast		24	CD	AL	8–24 [5]	3	5.8 ± 0.4	235 ± 27.1
	Fast		24	CS+E	AL	8–24 [5]	2	7.0 ± 0.9	317 ± 33.0
	Fast		24	CD+E	AL	8–24 [5]	3	8.0 ± 0.4 [3]	347 ± 22.6 [3]
D-3	Fast		24	CS	AL	24	2	3.8 ± 0.8	214 ± 27.0
	Fast		24	CD	AL	24	3	6.5 ± 0.6	339 ± 24.1 [4]
	Fast		24	CS+E	AL	24	2	7.6 ± 0.9	335 ± 22.8
	Fast		24	CD+E	AL	24	3	7.5 ± 0.6 [4]	341 ± 35.7

[1] CS = choline-supplemented diet; CD = choline-deficient diet; E = 0.25% DL-ethionine supplement; AL = ad libitum feeding; FF = force-feeding; rats weighed 60 to 74 g. Fast = 24 hour fast.
[2] Mean ± SE. All comparisons were made with CS group of each experiment.
[3] $P < 0.01$.
[4] $0.05 > P > 0.01$.
[5] Diet removed after 8 hours and animals were killed after 24 hours.

rats force-fed the CD diet containing 0.25% ethionine. Rats force-fed the CS diet with or without ethionine showed a greater (59 to 68%) decrease in liver lipid. In experiment 2 (table 4) rats were force-fed the CS or CD diet for 3 days. On the following morning rats of each group were then subdivided into three groups and were given 125 mg ethionine, 125 mg methionine or saline intraperitoneally 4 hours before killing. Analyses of liver lipids revealed little or no changes among the animals previously fed the CD or CS diet. In experiment 3 (table 4), groups of rats were force-fed for 3 days a CD or CS diet with or without ethionine. On the morning of day 4 some animals of each group were killed while others of the CD and CS groups were force-fed for 1 day the CD diet with ethionine as indicated in table 4. The results indicate no protective effect on liver lipid accumulation by the addition of ethio-

nine to the CD diet for 1 day. Thus, the results (table 4) indicate that ethionine supplementation to the CD diet will slowly (by 3 days) cause a decrease in the already accumulated hepatic lipids.

Because both methionine and adenine have been demonstrated to inhibit many of the acute effects of ethionine (3, 15), it was of interest to test whether either one of these compounds would alter the inhibitory effect of ethionine on choline-deficiency fatty liver. As a preliminary experiment we first determined what level of methionine and also what level of choline would protect against the fatty liver induced by force-feeding the CD diet for 3 days. Table 5 summarized the results which indicated that hepatic lipid accumulation

TABLE 4

Influence of ethionine or methionine treatment on liver lipid in rats fed a choline-deficient diet for 3 days

Exp. no.	Experimental conditions [1]						No. of rats	Liver lipid	
	Pretreatment			Treatment					
	Diet	Route	Duration	Diet	Route	Duration			
			days			*days*		%	*mg/liver/100 g body wt*
1	CD	AL	3	—	—	—	2	16.6 ± 2.9 [2]	891 ± 188.2 [2]
	CD	AL	3	CS	FF	3	4	8.2 ± 0.9	355 ± 34.5
	CD	AL	3	CD	FF	3	4	17.9 ± 2.1	872 ± 139.7
	CD	AL	3	CS+E	FF	3	3	7.5 ± 1.4	280 ± 46.0
	CD	AL	3	CD+E	FF	3	3	13.1 ± 1.7	509 ± 73.9
2	CD	FF	3	Sal.	IP	1/6	3	13.5 ± 4.1	635 ± 189.2
	CD	FF	3	Eth.	IP	1/6	4	13.0 ± 1.6	561 ± 78.2
	CD	FF	3	Meth.	IP	1/6	4	13.3 ± 1.5	595 ± 70.7
	CS	FF	3	Sal.	IP	1/6	3	4.5 ± 0.2	177 ± 12.0
	CS	FF	3	Eth.	IP	1/6	3	5.6 ± 0.3	209 ± 5.1
	CS	FF	3	Meth.	IP	1/6	3	4.4 ± 0.4	193 ± 28.8
3	CD	FF	3	—	—	—	4	9.8 ± 2.3	462 ± 94.5
		FF	3	CD+E	FF	1	3	10.1 ± 13.2	530 ± 107.9
	CS	FF	3	—	—	—	2	5.9 ± 0.6	295 ± 97.0
		FF	3	CD+E	FF	1	1	6.3	262
	CD+E	FF	3	—	—	—	3	7.2 ± 0.6	268 ± 30.8
	CS+E	FF	3	—	—	—	3	6.4 ± 0.7	250 ± 4.1

[1] CD = choline-deficient diet; CS = choline-supplemented diet; E = 0.25% DL-ethionine; Sal. = saline; Eth. = 125 mg DL-ethionine; Meth. = 125 mg DL-methionine; AL = ad libitum feeding; FF = force-feeding; and IP = intraperitoneal injection.
[2] Mean ± SE.

TABLE 5

Liver lipid of rats force-fed a choline-deficient diet with or without supplementation of choline or methionine for 3 days

Choline-deficient diet supplement		No. of rats	Liver lipid	
Choline	Methionine			
%	%		%	*mg/liver/100 g body wt*
0	0	3	12.9 ± 0.9 [1]	497 ± 45.6 [1]
0.02	0	4	11.7 ± 1.9	479 ± 60.1
0.06	0	3	7.4 ± 0.3	264 ± 20.4
0.12	0	3	6.5 ± 0.3	228 ± 5.1
0.60	0	4	5.3 ± 0.3	200 ± 1.4
0	0.1	4	8.1 ± 0.5	314 ± 15.7
0	0.2	4	7.0 ± 0.4	253 ± 15.3
0	0.4	3	6.3 ± 0.6	235 ± 15.8

[1] Mean ± SE.

occurred when animals were force-fed the CD diet without or with 0.02% choline or 0.1% methionine supplementation, but not when 0.06, 0.12 or 0.60% choline, or 0.2 or 0.4% methionine, was used. The latter effect of dietary methionine level is similar to that observed earlier in another study where a water-soluble chemically defined diet devoid of choline was used (13). Table 6 summarizes the results of one experiment (exp. 10) where groups of rats were force-fed for 3 days the CD diet without or with supplements of ethionine, methionine or ethionine and methionine. The results indicate that methionine partially protects against the ethionine-induced inhibition of choline-deficiency fatty liver. Hepatic lipid increased 80% by adding methionine to the CD diet containing ethionine. Table 6 also summarizes results of two experiments (indicated as exp. 11, table 6) in which the addition of 0.25% adenine sulfate to the CS or CD diet with supplemented ethionine did moderately elevate (51 and 41%, respectively) hepatic lipids in both groups force-fed for 3 days. Thus, methionine but not adenine seems to partially counteract the inhibitory effect of ethionine on choline-deficiency fatty liver.

Because ethionine is considered to be a competitive inhibitor of methionine, it was of interest to determine whether there were any alterations in the levels of hepatic free methionine in the experimental groups of animals. Table 7 presents data of the concentration and total levels of hepatic free methionine in two experiments. In both experiments (exps. 30 and 31), rats force-fed the CD diet for 3 days reveal a marked drop (75 to 81%); those fed the CD diet plus ethionine a 19 to 46% decrease; and those fed the CS diet plus ethionine a 39 to 50% decrease in total hepatic free methionine when compared with the total levels of animals fed the CS diet. Thus, it is apparent that the addition of ethionine to the CD diet accounts for an increase (116 to 315%) in total hepatic free methionine. A similar increase (91%) was found when ethionine was administered intraperitoneally instead of with the diet (exp. 30, table 7). To determine whether this increase of methionine was chemically important in altering the S-adenosylmethionine (SAM) or S-adenosylethionine (SAE) levels in the liver, SAE was measured in one experiment. Rats force-fed the CS diet supplemented with ethionine had 0.335 μmole SAE/g liver whereas those force-fed the CD diet plus ethionine had 0.641 μmole SAE/g liver.

In one experiment (exp. 31, table 7) ^{14}C-methyl-labeled methionine (1.47 mCi/mmole) was administered intraperitoneally to animals fed the CS or CD diet with or without ethionine for 3 days and killed 15 minutes later. In addition to hepatic free methionine levels, the specific activity of free methionine was determined (exp. 31,

TABLE 6

Liver weight and lipid of rats force-fed a choline-supplemented or choline-deficient diet with or without ethionine, adenine or methionine for 3 days

Exp. no.	Group [1]	No. of rats	Liver		
			Wt	Lipid	
			g/100 g body wt	*%*	*mg/liver/100 g body wt*
10	CD	3	4.66 ± 0.22 [2]	10.5 ± 1.1 [2]	495 ± 64.4 [2]
	CD + E	4	3.42 ± 0.05	5.9 ± 0.8	199 ± 22.5
	CD + M	3	4.78 ± 0.11	7.2 ± 0.6	345 ± 34.7
	CD + E + M	5	4.04 ± 0.11	8.7 ± 0.4	359 ± 44.0
11	CS	8	4.41 ± 0.10	4.5 ± 0.5	194 ± 26.8
	CD	8	4.60 ± 0.13	11.3 ± 2.1	508 ± 113.4
	CS + E	6	3.54 ± 0.38	4.7 ± 0.8	168 ± 34.4
	CD + E	8	3.16 ± 0.08	5.0 ± 0.6	169 ± 7.7
	CS + E + A	6	3.71 ± 0.20	6.6 ± 0.7	254 ± 31.3
	CD + E + A	4	3.38 ± 0.06	7.1 ± 0.2	239 ± 9.2

[1] CS = choline-supplemented diet; CD = choline-deficient diet; E = 0.25% DL-ethionine supplement; A = 0.25% adenine sulfate supplement; M = 0.25% DL-methionine supplement.
[2] Mean ± SE.

TABLE 7

Hepatic free methionine levels and ^{14}C-methyl labeled methionine incorporation into hepatic proteins of rats force-fed a choline-deficient or choline-supplemented diet with or without ethionine for 3 days

Exp. no.	Group [1]	No. of rats	Sex	Liver free methionine [2]		Methionine radioactivity [2,3]		Liver protein radioactivity [2]	
				μmoles/g liver	*μmoles/liver/ 100 g body wt*	*cpm/μmole methionine*	*cpm/liver/ 100 g body wt*	*cpm/mg protein*	*cpm/liver/ 100 g body wt*
30	CS	4	F	0.074	0.306				
	CD	2	F	0.017	0.077				
	CS+E	5	F	0.040	0.152				
	CD+E	5	F	0.049	0.166				
	CS+E [4]	5	F	0.034	0.123				
	CD+E [4]	3	F	0.040	0.147				
31	CS	2	M	0.066	0.304	77	23.4	23.8	18.031
	CD	2	M	0.012	0.059	1522	89.8	34.0	25.286
	CS+E	2	M	0.057	0.187	100	18.7	39.3	21.960
	CD+E	2	M	0.079	0.245	238	58.3	44.3	23.415

[1] CS = choline-supplemented diet; CD = choline-deficient diet; E = ethionine supplement.
[2] Each value is from pooled specimens of livers.
[3] ^{14}C-methyl labeled DL-methionine (1.47 μCi/μmole) administered intraperitoneally 15 minutes before killing.
[4] DL-Ethionine (22.5 mg/rat per day) administered intraperitoneally twice daily.

table 7). The high value in the CD group was at least partly related to the low level of free methionine in the liver. It is important to note, however, that the value was also high in the CD plus ethionine group. In this experiment ^{14}C incorporation into hepatic protein was also determined (exp. 31, table 7). The results indicate essentially similar degrees of incorporation in liver protein in all four groups. Since the specific activities of ^{14}C-methionine were so different in the four groups, it is difficult to interpret the true incorporation into hepatic protein. It does indicate, however, that the supplementation of ethionine in these experiments does not inhibit hepatic protein synthesis.

DISCUSSION

Much information has accumulated concerning the pathogenesis of the acute induction of fatty liver due to choline-deficiency and to ethionine (2–5). Nonetheless, the present observations that ethionine supplementation inhibits the fatty liver due to choline-deficiency are difficult to explain. In an attempt to gain some insight into possible mechanism, we have considered several aspects in our search for clues. They are: 1) the possible influence of dietary components on the action of ethionine when it is supplemented to the diet; 2) the possible role of changes in hepatic choline oxidase activity; and 3) the possible importance of alterations in levels of hepatic free methionine.

Fatty liver can be induced rapidly when ethionine is administered to fasted female rats but this is prevented when methionine is supplemented, alone or in dietary protein (3, 16). Therefore, the effect of ethionine when administered alone to fasted animals can be quite different from when given together with diet. Prefeeding animals with a purified casein diet before administering ethionine has been reported to protect against some of the acute effects due to ethionine.[7] Likewise, in our present study we observed that prefeeding the CS diet for 1 day led to less liver lipid rise after 1 day of feeding the CS diet supplemented with ethionine than following a 1-day fast

[7] Fisher, M. M., and K. H. Shull 1968 Dietary influences on the hepatic response to acute ethionine administration. Federation Proc., 27: 606 (abstract).

(table 3); and the minor rise in hepatic lipid that developed, due to ethionine addition to the CS or CD diet after 1 day, disappeared with further feeding the same diets for 2 or 3 days (table 3). Thus the effect of diet on ethionine action is clear, yet how diet is responsible, whether through methionine, calories, or the like, is not clear. Therefore, it is difficult to evaluate whether much of the information learned from the acute administration of ethionine to fasted animals (3) can be applied to studies in which ethionine is administered along with the diet.

In an earlier study (12) hepatic choline oxidase activity was investigated in a number of species, and a possible correlation between the level of this enzyme in each species and the ease of induction of choline-deficiency fatty liver was considered. Because choline oxidase provides the only known mechanism for irreversible biological degradation of choline in higher animals, it was thought that a reduced level of the enzyme might help to conserve the body store of choline and could make it much more difficult to induce a choline deficiency. Earlier it was reported that hepatic choline oxidase is greatly reduced in rats receiving dietary ethionine (17). In our present study we likewise found that the addition of ethionine to the CS or CD diet caused a reduction in hepatic choline oxidase activity. It is conceivable that this reduction in choline oxidase activity by ethionine conserves some choline, and therefore, may play a role in the ethionine inhibition of choline-deficiency fatty liver.

Because dietary methionine, even at relatively low levels, has been demonstrated to protect against choline-deficiency fatty liver, the hepatic free methionine levels were determined in two of our experiments (table 7). The results indicated that the addition of ethionine to the CD diet caused an elevation in hepatic free methionine, bringing the level close to that found in rats fed the CS diet. Other reports have revealed an elevation of free methionine in the livers of rats treated with ethionine (18, 19). It was reported earlier, however, that ethionine decreased the amount of transmethylation from methionine to choline by about 20% (20). Yet this large elevation in hepatic free methionine due to ethionine could possibly be important in preventing the choline-deficiency fatty liver.

Our present observations raise the question whether the addition of dietary ethionine will protect against other experimental forms of fatty liver, particularly those considered to be related to nutritional disturbances. In a few preliminary experiments [8] the addition of ethionine to a purified diet containing orotic acid (21) partially protected against the lipid accumulation induced by orotic acid (21–23). Also, it has recently been reported that the supplementation of orotic acid to a choline-deficient diet reduced the levels of hepatic triglycerides in choline-deficient rats (24). Because fatty livers due to ethionine and to orotic acid can both be prevented by supplementing adenine (23, 25), a possibly related disturbance in mechanism has been considered (26). It becomes difficult, however, to delineate at this time how ethionine may act to diminish the lipid accumulation due to orotic acid or to choline deficiency. Because ethionine has been demonstrated to affect organs other than the liver, including endocrine glands (3), it is possible that it may act indirectly through hormonal changes. Yet, it is of interest that ethionine, which has been reported to demonstrate sex differences in some responses (3), inhibited the choline-deficiency fatty liver in both male and female rats.

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