Effect of Bengal Gram on Experimentally Induced High Levels of Cholesterol in Tissues and Serum in Albino Rats

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ABSTRACT Bengal gram (*Cicer arietenum*), a protein-rich cereal which forms the staple diet of people of low socioeconomic status in Northern India, was found to have a marked hypocholesterolemic effect in cholesterol-cholic acid-fed rats. It prevented, as well as reversed, the experimentally induced high levels of cholesterol in both tissues and serum. Both its protein and fat fractions were found to cause this effect. Increased excretion of cholesterol as bile acids and neutral sterols and decreased synthesis in liver are the probable mechanisms of its action.

An epidemiological survey carried out by Mathur et al. (1) in Agra, India, revealed that people of low socioeconomic status, whose staple diet was a protein-rich cereal, Bengal gram,[1] had much lower levels of serum cholesterol and a lower incidence of ischemic heart disease than others not consuming that diet. Hypocholesterolemic properties of Bengal gram were also observed during the course of preliminary studies of the effect of dietary fats and proteins on serum cholesterol in experimental animals (2). These observations prompted us to study in greater detail the effect of Bengal gram on serum and tissue lipids in albino rats fed a hypercholesterolemic diet.

EXPERIMENTAL

Bengal gram was fed in the form of powder to investigate the effect of whole gram. It was extracted with butyl alcohol for 24 hours, and the defatted gram and lipid extract of gram thus obtained were studied separately.

A total of 170 male albino rats was used. The animals were divided into 7 groups, for a 12-week study. Groups 1, 3, 4, 6 and 7 consisted of 20 rats each, and groups 2 and 5, 35 rats each.[2] The composition of the diets fed the various groups is shown in table 1. All the animals were fed a hypercholesterolemia-inducing diet. Group 1 rats served as controls and received the hypercholesterolemia-inducing diet alone throughout the experiment. Substitution with whole gram, defatted gram and lipid extract of gram was made in groups 2, 3 and 4, respectively, from the beginning keeping the diets isocaloric. Rats of groups 5, 6 and 7 were fed a hypercholesterolemia-inducing diet alone for 6 weeks for preliminary induction of hypercholesterolemia after which whole gram, defatted gram and lipid extract of gram replacements were made for the following 6 weeks.

At the end of the first 6 weeks of study, the rats of groups 2, 3 and 4 were killed, along with one-half the number of controls from group 1. The remaining control rats of group 1 were killed along with rats of groups 5, 6 and 7 at the end of 12 weeks.

Each rat was numbered and weighed weekly. A record of the daily dietary consumption was maintained. Forty-eight hours prior to the time of killing, each rat was put in a metabolic cage and the feces collected. At the time of killing, 4 to 6

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[1] Bengal gram (*Cicer arietenum*) belongs to the family Leguminosae. Each 100 g provide: water, 9.8 g; protein, 17.1 g; fat, 5.3 g; minerals (ash), 2.7 g; crude fiber, 3.9 g; carbohydrate, 61.2 g; calcium, 0.19 g; phosphorus, 0.24 g; thiamine, 0.3 mg; and vitamin A, 316 IU in the form of carotene. Its protein is rich in the essential amino acids except for tryptophan and methionine, and the percentage composition of the fatty acids present in the lipid is: oleic acid, 52.1; linoleic acid, 38; myristic acid, 2.74; palmitic acid, 5.11; and stearic acid, 2.05. β-Hydroxysterols and phospholipids are also present. Bengal gram serves as the chief source of protein for people of lower socioeconomic status, who use it in bread made of gram flour. It can be made into soup, curry (boiled or fried), and candy, and can be worked into a complete dinner for use by people of any socioeconomic status. Bengal gram is extensively cultivated in Northern India, forming one of the major crops, and is a very inexpensive cereal.

[2] Twenty-six rats died during the experiment.

TABLE 1

Composition of diets fed to various groups of rats

Diet	Hydrogenated ground nut oil	Casein	Sucrose	Whole gram flour	Defatted gram flour	Fat-extract of gram	Salt [1] mixture	Cholesterol	Cholic acid	Choline chloride	Vitamin mixture [2]
	%	%	%	%	%	%	%	%	%	%	%
Control [3]	20	15	60	—	—	—	3.4	1	0.2	0.3	0.1
Control + whole gram	20	—	—	75	—	—	3.4	1	0.2	0.3	0.1
Control + defatted gram flour	20	—	5	—	70	—	3.4	1	0.2	0.3	0.1
Control + fat extract of gram	20	15	57	—	—	3	3.4	1	0.2	0.3	0.1

[1] Hawk, P. B., B. L. Oser and W. H. Summerson 1954 Practical Physiological Chemistry, ed. 13. McGraw-Hill Book Company, New York, p. 1375.
[2] The percentage composition of the vitamin mixture was: vitamin A concentrate (200,000 units/g), 9; vitamin D concentrate (400,000 units/g), 0.5; α-tocopherol, 10; ascorbic acid, 9; inositol, 10; menadione, 4.45; p-aminobenzoic acid, 10; niacin, 9; riboflavin, 2; pyridoxine·HCl, 2; thiamine·HCl, 2; Ca pantothenate, 6; biotin, 0.04; folic acid, 0.18; and glucose, 25.83.
[3] Hypercholesterolemia-inducing diet.

cm [3] of blood were drawn by direct cardiac puncture and the liver was removed for analysis.

Serum total, free and ester cholesterol were estimated by the method of Zak et al. (3), serum lipid phosphorus by that of Fiske and Subarrow (4) and the cholesterol content of α- and β-lipoproteins by the method of Langan et al. (5).

The liver was treated with alcohol acetone mixture for 8 hours, in flasks fitted with condensers. This tissue extract was used to estimate total cholesterol by the same methods used for the serum. Estimation of cholic and deoxycholic acid in feces was carried out by the method of Mosbach et al. (6). The sulfhydryl content and coenzyme A content of liver were estimated in 8 animals each of groups 1, 2, 3 and 4 by the methods of Grunert and Phillips (7) and Novelli et al. (8), respectively.

RESULTS AND DISCUSSION

No statistically significant variation ($P > 0.05$) was noted in the weight gain and the daily dietary consumption in the various groups of rats.

The serum lipid levels of groups 1, 2, 3 and 4 are shown in table 2. The serum total cholesterol levels for the animals of groups 2, 3 and 4 which were fed whole gram, defatted gram and lipid extract of gram, respectively, were significantly lower ($P < 0.001$) than those for the control rats. Lipid phosphorus concentration was unaffected. Both the free cholesterol and the ester cholesterol were significantly lower ($P < 0.001$), as were the values for the total cholesterol-to-lipid phosphorus ratio ($P < 0.01$). The cholesterol content of α- and β-lipoprotein in serum was also significantly lower ($P < 0.001$) for all the rats receiving Bengal gram and its fractions.

TABLE 2

Serum lipids of rats of groups 1, 2, 3 and 4

Group no. [1]	Dietary supplement	Total cholesterol	Free cholesterol	Ester cholesterol	Lipid phosphorus	Cholesterol content	
						α-Lipoprotein	β-Lipoprotein
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
1	Control (19) [2]	174.7 ± 32.3 [3]	73.5 ± 23.2	101.2 ± 13.3	8.7 ± 1.1	135.7 ± 25.3	36.1 ± 7.9
2	Whole gram (31)	106.0 ± 22.7	40.9 ± 15.1	65.0 ± 22.5	8.6 ± 5.0	84.3 ± 9.2	20.3 ± 4.4
3	Defatted gram (17)	111.3 ± 26.3	43.1 ± 22.3	68.1 ± 22.8	10.8 ± 2.3	89.1 ± 17.8	21.3 ± 6.5
4	Lipid extract of gram (15)	89.2 ± 14.3	37.1 ± 13.1	51.1 ± 12.9	8.3 ± 2.9	70.5 ± 15.8	14.9 ± 4.1

[1] After 6 weeks of study.
[2] Numbers in parentheses indicate number of rats per group that survived.
[3] Mean ± SD.

The serum lipids of groups 5, 6 and 7 are compared with those of the controls in table 3. The decrease in the serum total cholesterol, free and ester cholesterol follows the same pattern as that for groups 2, 3 and 4.

Bengal gram and its fractions were, therefore, found to be effective in lowering elevated serum cholesterol levels as well as preventing an elevation when fed together with a hypercholesterolemia-inducing diet.

In table 4 is shown the fecal excretion of cholesterol end products. The fecal excretion of bile acids, both cholic and deoxycholic acid, were found to be significantly increased ($P < 0.01$) in the animals which received whole gram (groups 2 and 5) and lipid extract of Bengal gram (groups 4 and 7) compared with that of control rats. Byers and Friedman (9) and Portman and Sinisterra (10) have reported similar observations after administration of unsaturated lipids. An increased excretion of cholesterol as bile acid in feces might therefore be one of the possible mechanisms of the hypocholesterolemic action of Bengal gram and its lipid fraction.

The possibility of some active principle in addition to the unsaturated fatty acids responsible for hypocholesterolemic action in the lipid extract can not be excluded since the amount of unsaturated fats, β-hydroxysterols and phospholipids in the lipid extract was small. The reasons for the difference in the behavior of rats of group 3 and 6 with respect to bile acids is not clear.

In table 5 are shown the results of total cholesterol sulfhydryl content and coenzyme A concentration in the liver of animals of groups 1, 2, 3 and 4.

The total cholesterol was appreciably lower in livers of groups 2, 3 and 4 compared with that of control rats. The sulfhydryl content and the coenzyme A concentration in the liver of animals which received Bengal gram and defatted gram were also significantly lower ($P < 0.001$) than those of the control animals.

TABLE 3

Serum lipids of rats of groups 1, 5, 6 and 7

Group no.[1]	Dietary supplement	Total cholesterol	Free cholesterol	Ester cholesterol	Lipid phosphorus	Cholesterol content	
						α-Lipoprotein	β-Lipoprotein
		mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
1	Control (19) [2]	176.7 ± 15.2 [3]	69.9 ± 27.4	106.4 ± 22.0	9.5 ± 2.4	136.1 ± 34.7	38.2 ± 5.9
5	Whole gram (31)	107.0 ± 35.3	41.3 ± 12.9	65.6 ± 29.7	9.2 ± 3.2	85.4 ± 23.4	20.6 ± 6.4
6	Defatted gram (18)	117.5 ± 29.3	42.8 ± 15.1	74.7 ± 22.7	7.8 ± 1.5	86.8 ± 8.0	19.8 ± 5.2
7	Lipid extract of gram (13)	95.9 ± 16.2	41.7 ± 11.2	54.1 ± 14.9	8.1 ± 2.6	80.2 ± 14.0	14.1 ± 2.6

[1] After 12 weeks of study.
[2] Numbers in parentheses indicate number of rats per group that survived.
[3] Mean ± SD.

TABLE 4

Fecal bile acid values for various groups of rats

Group no.	Dietary supplement	Cholic acid	Deoxycholic acid	Total bile acids
		mg/kg body wt/24 hr	*mg/kg body wt/24 hr*	*mg/kg body wt/24 hr*
1 [1]	Control (19) [2]	3.2 ± 1.1 [3]	2.7 ± 1.1	5.9 ± 2.9
1 [4]	Control (19)	3.3 ± 1.4	3.0 ± 1.2	6.39 ± 2.7
2 [1]	Whole gram (31)	5.7 ± 1.3	3.2 ± 2.0	8.7 ± 2.0
3 [1]	Defatted gram (17)	4.3 ± 1.3	3.7 ± 1.0	8.1 ± 2.1
4 [1]	Lipid extract of gram (15)	8.6 ± 2.4	7.7 ± 2.3	16.3 ± 3.4
5 [4]	Whole gram (31)	5.9 ± 1.2	5.5 ± 1.1	11.5 ± 2.4
6 [4]	Defatted gram (18)	3.4 ± 1.0	3.0 ± 0.7	6.53 ± 1.3
7 [4]	Lipid extract of gram (13)	8.0 ± 2.4	7.0 ± 2.2	15.0 ± 4.5

[1] After 6 weeks of study.
[2] Numbers in parentheses indicate number of rats per group that survived.
[3] Mean ± SD.
[4] After 12 weeks of study.

TABLE 5

Liver sulfhydryl, coenzyme A content and total cholesterol concentration in rats of groups 1, 2, 3 and 4

Group no.	Dietary supplement	Sulfhydryl content	Coenzyme A conc	Liver total cholesterol
		mg/100 g	*mg/100 g*	*mg/100 g*
1	Control (19) [1]	304.5 ± 56.9 [2]	130.4 ± 22.9	1006 ± 146.5
2	Whole gram (31)	164.0 ± 26.2	87.0 ± 12.1	767 ± 195.4
3	Defatted gram (17)	149.9 ± 24.5	92.5 ± 21.6	583 ± 165.9
4	Lipid extract of gram (15)	279.0 ± 53.3	127.8 ± 22.7	516 ± 118.2

[1] Numbers in parentheses indicate number of rats per group that survived.
[2] Mean ± SD.

In view of the observation of Olson and Stare (11) that a decrease in the tissue content of coenzyme A means reduced capacity for acetylation, and reports of Bagchi et al. (12) that serum cholesterol levels in albino rats correlate closely with sulfhydryl content of liver and that coenzyme A, which has a functional sulfhydryl group, plays an important role in the synthesis of cholesterol, we presume that in the present experiment, the biosynthesis of cholesterol was inhibited in rats fed whole gram and defatted gram.

The hypocholesterolemic action of Bengal gram has been confirmed by the present studies with rats. If these studies can be reproduced in human subjects, Bengal gram will prove to be an inexpensive and practical hypocholesterolemic agent.

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Influence of Dietary Cadmium Level and Supplemental Zinc on Cadmium Toxicity in the Bovine [1,2,3]

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ABSTRACT Growth rate, feed consumption, water intake, and testicle development decreased progressively as the concentration of Cd in the diet increased. Blood hemoglobin decreased slightly when 40 to 160 ppm Cd in the diet were consumed, but increased sharply when higher levels of Cd (640 and 2560 ppm) were fed. Calves fed 640 or 2560 ppm Cd exhibited unthrifty appearance; rough hair coat; severe body dehydration; dry and scaly skin; loss of hair; mouth lesions; edematous, shrunken, and scaly scrotum; sore and enlarged joints; impaired sight; and liver and kidney damage. Feeding 40 to 160 ppm of Cd from 9 to 20 weeks of age resulted in few clinical symptoms. There was 100% mortality in calves given 2560 ppm Cd, with death occurring in 2 to 8 weeks. At the 640 ppm level there was some mortality. When Cd was removed from the diet, a very severely affected calf recovered to normal appearance with a substantial quantity of live, but mostly abnormal, sperm later obtained from the epididymis. The data suggest that addition of zinc partially offset the effects of Cd on calf performance. Kidneys and livers contained the highest concentration of Cd.

Dietary cadmium (Cd) is highly toxic to several species of monogastric animals (1–9). A part of this effect is due to it being a strong antimetabolite for zinc (10, 11). Thus Cd could be a very useful tool in studying certain aspects of zinc nutrition. The toxicity syndrome and the levels of Cd required to give specific effects have been investigated partially in a number of species of monogastric animals. However, no previous studies have been found in which Cd toxicity was studied in ruminants.

The primary objective of the studies herein reported was to characterize the Cd toxicity syndrome in the bovine and to relate level of Cd to the degree of toxicity. A secondary objective was to obtain some information on the cadmium-zinc relationship in cattle; especially to determine whether supplemental zinc would tend to alleviate the Cd toxicity.

EXPERIMENTAL

Twenty-four Holstein and 8 Jersey bull calves were used in an experiment which consisted of a standardization phase (second through eighth week of age) immediately followed by a treatment period of 12 weeks' duration. The calves were allowed to nurse their dams for one day, then fed 3.2 kg of whole milk/day for 2 days. From day 4 through day 38 the Holsteins were given daily, 0.41 kg of dry commercial milk replacer which was mixed with 9 parts water. The Jerseys were fed 0.32 kg/day until 42 days old. The calves were maintained in individual wooden pens with elevated bottoms and bedded with pine shavings. Plastic buckets were used to feed both milk and water.

From the second through the eighth week of age all calves were fed the practical-type basal ration ad libitum (table 1) which was comparatively low in zinc. It contained 88.6% dry matter and the following by analyses on a dry-matter basis: zinc, 27.4 ppm; Cd, 0.32 ppm; crude protein, 14.8%; crude fiber, 14.1%; ether extract, 4.2%; and ash, 8.2%. The calculated calcium content was 2.2%.

At the beginning of their ninth week of age, the calves were assigned at random to one of 8 experimental rations which were fed ad libitum for 12 weeks. The rations were: a) basal (table 1); b) basal + 100 ppm zinc; c) basal + 40 ppm cadmium; d) basal + 40 ppm cadmium + 100 ppm zinc; e) basal + 160 ppm cadmium; f)

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[2] Most of the data in this paper are taken from a thesis submitted by the senior author.

[3] Supported in part by a Graduate Assistantship from the Graduate School of the University of Georgia to the senior author.

TABLE 1

Composition of basal diet

	kg/100 kg
Citrus pulp	35.5
Beet pulp	20.8
Corn	11.6
Soybean meal	6.2
Coastal bermudagrass pellets	19.3
Urea (42%) [1]	0.8
Dicalcium phosphate	1.7
Salt and trace minerals [2]	0.8
Marble dust	1.5
Stabilized fat	1.5
Vitamins and antibiotics [3]	0.3

[1] Procadian Urea Animal Feed Mixture, Allied Chemical Company, Atlanta, Ga.

[2] Composition: NaCl, 100 kg; $MnSO_4 \cdot H_2O$, 696 g; $Fe_2(SO_4)_3 \cdot nH_2O$ (78.5% $Fe_2(SO_4)_3$ by assay), 441 g; $CuSO_4 \cdot 5H_2O$, 84.6 g; $CoCl_2 \cdot 6H_2O$, 4.4 g; KI, 11 g.

[3] Aurofac 10, 255.7 g (chlortetracycline hydrochloride, 22 g/kg). American Cyanamid Company, New York. Vitamin A, 85.1 g (20,000 IU/g) and vitamin D, 3.3 g (27,000 IU of D_3/g) Dawes Laboratories Inc., Chicago.

basal + 160 ppm cadmium + 100 ppm zinc; g) basal + 640 ppm cadmium; and h) basal + 2560 ppm cadmium. The zinc was added as ZnO powder to a pre-mix. To avoid dust the Cd, as $CdCl_2$, was dissolved in water and poured over the feed as it was mixed.

Clinical examinations were made weekly. Feed and water consumption were measured 7 days every other week. Body weights were taken for 3 days at the beginning and end of the standardization and treatment phases, and one day every second week during the experiment. Blood samples were collected from the jugular vein bi-weekly and potassium oxalate was used to prevent coagulation. Hemoglobin levels were determined by the method of Cohen and Smith (12). Testicle measurements were made bi-weekly through the scrotum with a vernier caliper as described previously (13). All animals were castrated at the completion of the experiment and the testicles trimmed to kidney shape and weighed. Tissue samples were taken and necropsy examinations made on all animals that died. One control and one that received 100 ppm zinc were put to death by euthanasia and used for analyses and postmortem studies. The samples were frozen until analyzed for Cd and zinc.

The tissues were sampled in the following manner: a) incisors 1 and 2, extracted and washed with redistilled water; b) liver, 2 g from the central portion; c) spleen, 2 g from the tip; d) kidney, one gram from the cortex; e) femur, 2 g of compact bone (cortex) from the diaphysis; the bone was cooked in an autoclave before sampling to remove any contaminating flesh; f) skin, 2 g from the neck—the skin was tacked to a board, warmed, lathered with shaving cream, and shaved before sampling; g) hair, 2 g of brushed, clean, but unwashed, black hair from the rib area; and h) testicles, the visceral layer and the tunica albuginea of the frozen testicles were removed, and 2-g cross sections of the remaining tissues analyzed.

Dry matter was determined on the tissues prior to digestion for zinc and Cd analyses by the atomic absorption spectrophotometric procedure (14). Digestion was by the nitric acid-perchloric acid method.

RESULTS AND DISCUSSION

Growth, feed and water consumption. The 4 calves fed 2560 ppm Cd did not grow, with deaths occurring at 2, 3, 5 and 8 weeks (fig. 1). There was a reduction in growth, feed intake, and water consumption of those fed as much as 160 ppm Cd without the addition of zinc (fig. 1, 2, and 3) and (table 2). One of the 4 calves died after receiving 640 ppm for 6 weeks. The 40-ppm level appeared to depress these measures of calf performance; however,

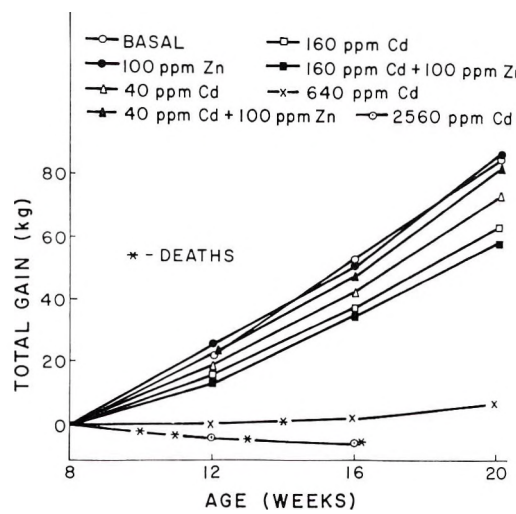

Fig. 1 Average total body weight gains for calves fed different levels of Zn and Cd.

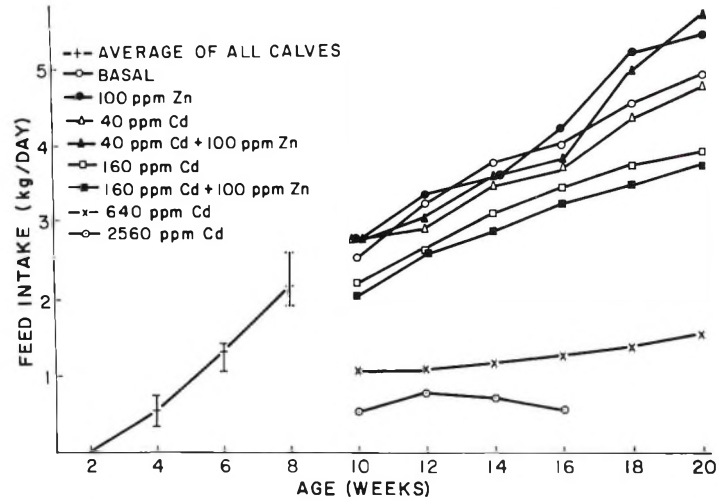

Fig. 2 Average daily feed consumption of calves fed different levels of Zn and Cd (unadjusted).

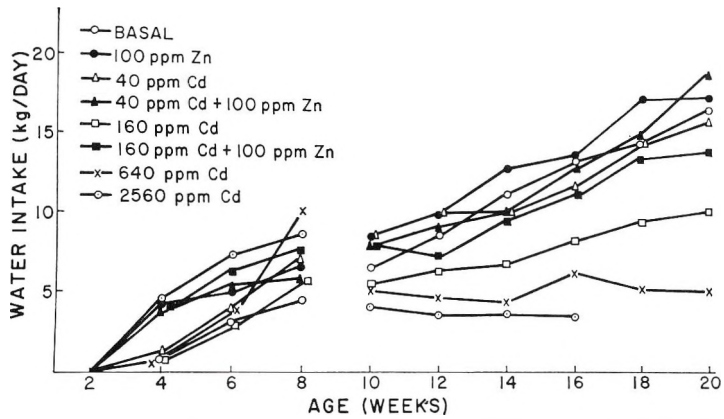

Fig. 3 Average daily water intake for calves fed different levels of Zn and Cd (unadjusted).

the degree of the effect was too small for statistical significance.

The results of this experiment suggest that calves can tolerate considerably higher concentration of Cd in the diet than rats, turkeys, or chicks (1, 4, 17, 18). If calculated per gram of tissue, the higher tolerance of calves for Cd relative to other species might not be so marked. However, most of the previous studies do not present sufficient information to make possible such a comparison. In rats, Wilson et al. (4) and Johns et al. (1) observed a slight growth reduction with 62 ppm Cd and death with 500 ppm. As little as 20 ppm depressed growth in turkey poults (17). Hill et al. (18) reported that the level of copper and iron modified the amount of Cd required to obtain a given effect. However, regardless of level of other factors, all the chicks given 400 ppm Cd died.

There was an immediate reduction in feed consumption for all the groups fed as much as 160 ppm Cd. This appeared the first day the Cd-containing diet was fed and suggests that the Cd was adversely affecting palatability.

Hemoglobin. Blood hemoglobin values decreased progressively as the level of Cd was increased from zero to 160 ppm (table 2). However, at higher levels of Cd, hemoglobin values were increased. Those fed 640 ppm had significantly higher values than those given 160 ppm Cd. The aver-

TABLE 2

Weight gain, feed and water consumption, blood zinc, and hemoglobin means [1]

Weight gains, kg/calf/day								
Treatment no.: [2]	7	6 [3]	5	3	4	1	2	S_x [4]
Means:	− 0.09	0.72	0.73	0.87	0.96	1.04	1.08	0.07
Feed consumption, kg/calf/day								
Treatment no.: [2]	7	5	6	3	4	2	1	S_x [4]
Means:	0.92	2.93	3.20	3.43	3.68	3.80	3.86	0.21
Water consumption, kg/calf/day								
Treatment no.: [2]	7	5	6	4	3	2	1	S_x [4]
Means:	4.42	8.91	9.44	11.53	12.01	12.67	12.78	1.02
Blood zinc, μg/ml								
Treatment no.: [2]	7	5	3	6	4	1	2	S_x [4]
Means:	1.42	1.80	1.94	2.16	2.22	2.23	2.45	0.11
Hemoglobin, g/100 ml								
Treatment no.: [2]	5	3	6	4	2	1	7	S_x [4]
Means:	7.93	8.70	8.84	8.96	9.04	9.17	9.37	0.42

[1] Adjusted for differences for the same measurement during the 2 to 8 week standardization period by covariance procedures (15).

[2] Treatment no. 1, control; treatment no. 2, 100 ppm Zn; treatment no. 3, 40 ppm Cd, treatment no. 4, 40 ppm Cd + 100 ppm Zn; treatment no. 5, 160 ppm Cd; treatment no. 6, 160 ppm Cd + 100 ppm Zn; treatment no. 7, 640 ppm Cd.

[3] Values underlined by the same line are not significantly different ($P = 0.05$) (16).

[4] SE of adjusted treatment mean (16 degrees of freedom).

age hemoglobin value at the last bleeding prior to death for those fed 2560 ppm was 12.1. These high hemoglobin levels for calves fed this treatment and the 640 ppm can reasonably be attributed to the severe body dehydration these animals exhibited as a result of Cd toxicity.

The effects of Cd administration on blood hemoglobin are not consistent in the published literature. In some instances toxicity has reduced hemoglobin values, whereas in others elevated values have been observed (2, 3, 7, 8, 18, 19).

It appears that low-to-moderate levels of Cd tend to reduce hemoglobin values, whereas high amounts of Cd may cause an increase. The levels of certain other nutrients have an influence (4). Hill et al. (18) have shown that hemoglobin values are reduced more in the absence of sufficient copper.

Clinical and necropsy examinations. The general symptoms of toxicity for the animals receiving the 640- and 2560-ppm levels of Cd were unthrifty appearance; rough hair coat; severe body dehydration; dry and scaly skin; loss of hair from legs, thighs, chest floor, and brisket; mouth lesions; edematous, shrunken, and scaly scrotum; sore and enlarged joints; impaired sight; extreme emaciation; and some atrophy of rear leg muscles. This atrophy is apparently due to the severe weight loss. Some of these symptoms are shown in figures 4 and 5.

The average time between initiation of treatment and occurrence of the various toxicity symptoms for the animals receiving the 640 and 2560 ppm levels of Cd is shown in table 3. All of these symptoms except edema of the scrotum occurred earlier among the animals that received 2560 ppm Cd. However, the symptoms were more severe in the animals that received the 640 ppm Cd. This is probably due to their intake of Cd over a longer period than those fed 2560 ppm Cd, thereby allowing more time for the symptoms to develop. In addition to the symptoms listed above, after about 40 days on treatment, calves fed the 640 ppm Cd diet developed skin which was easily injured. Thus bleeding resulted from accidently received minor injuries on their legs.

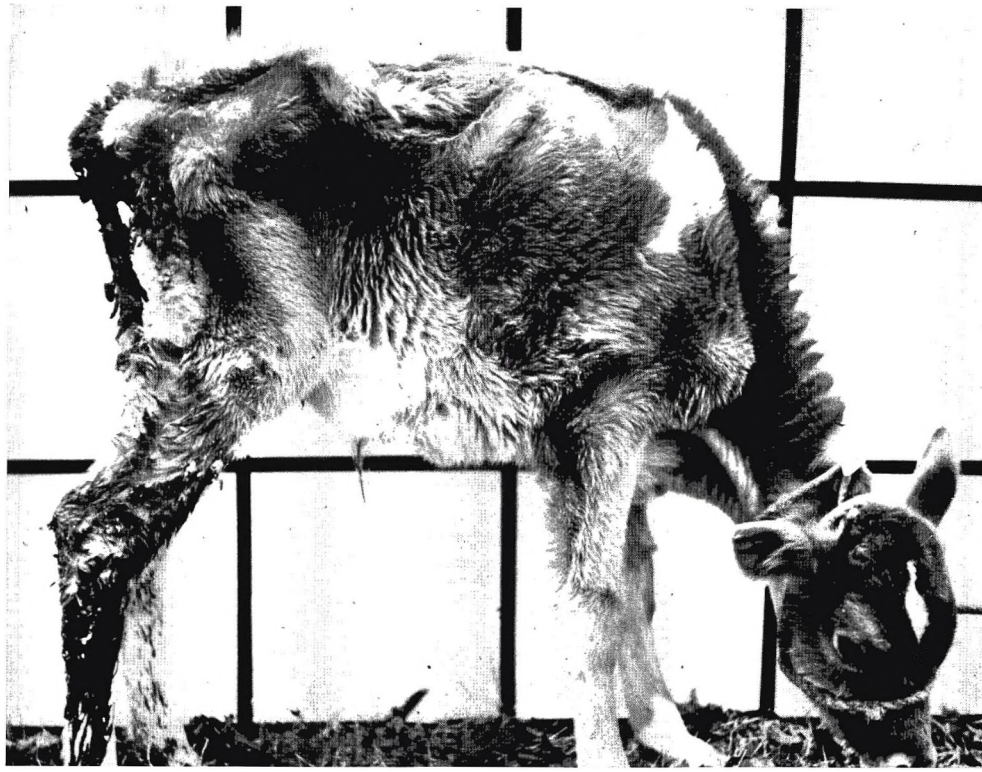

Fig. 4 Calf receiving diet containing 2560 ppm Cd for 14 days, age 10 weeks.

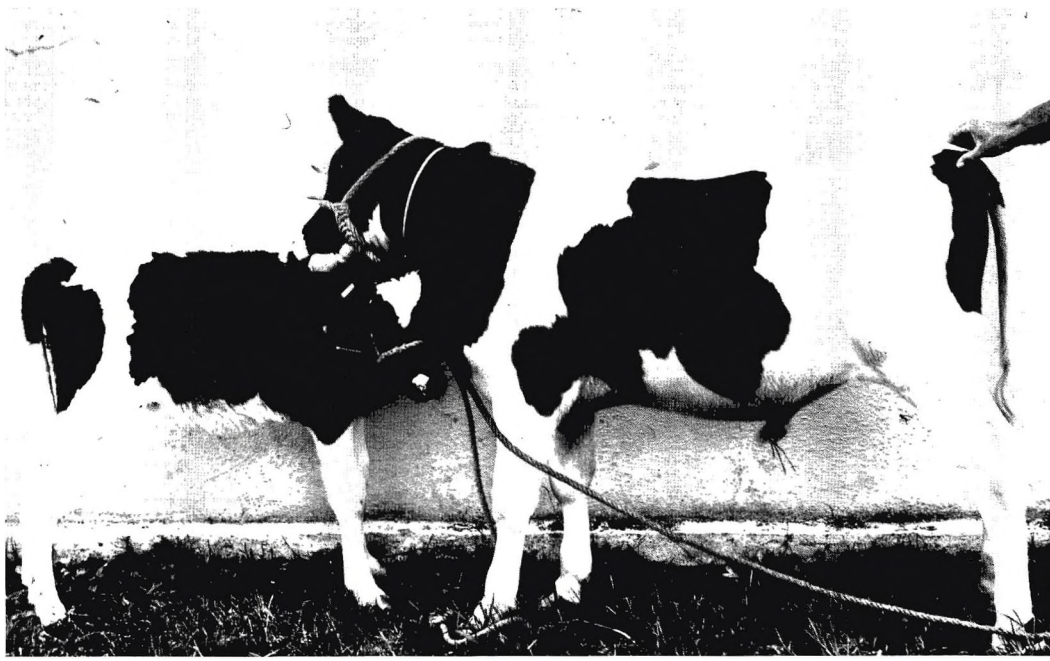

Fig. 5 Calf at left received diet containing 640 ppm Cd. Animal on right was control. Body weights when picture was taken at 18 weeks were 57 kg and 163 kg. Both weighed 45 kg at birth and were fed the same diet for first 8 weeks.

TABLE 3

Average days between initiation of treatment and occurrence of various toxicity symptoms for calves receiving 640 and 2560 ppm Cd in their diet [1]

Symptom	Treatment	
	640 ppm Cd	2560 ppm Cd
	days	
Edema in scrotum	20	28
Dry and scaly skin	22	19
Mouth lesions	33	16
Scaly scrotum	36	— [2]
Sore or enlarged joints	41	20
Loss of hair	44	25
Impaired sight	46	17
Atrophy of muscles [3]	64	17

[1] The average number of days was reduced by 3 to compensate for symptoms appearing between the weekly examinations.
[2] Symptom did not occur.
[3] Apparently due to the severe weight loss.

Of the calves receiving the 160 ppm Cd in the diet, only one developed mouth lesions (84 days after initiation of treatment). Two others developed edema in the scrotum after being fed the Cd diet an average of 54 days. Two calves fed 40 ppm Cd in the diet developed edema in the scrotum at 15 and 19 weeks of age. No other clinical toxicity symptoms were observed.

Relatively little information concerning clinical symptoms of Cd toxicity has been found. Ginn and Volker (3) have reported that rats receiving 50 ppm Cd in their drinking water developed dry and scaly skin and tails with thinning of the hair, especially in the posterior portion of the dorsum. Bleaching of the incisors of rats was noted in cases of chronic Cd toxicity (3, 5). In animals that vomit, such as cats, the chief effect of the oral administration of high levels of Cd is emesis (6).

Postmortem examinations indicated that the liver and kidneys were the organs in calves most affected by Cd feeding. The 2560-ppm level resulted in more serious damage to these organs than the 640-ppm level. Reports by Prodan (8) and Wilson et al. (2) show that these were the organs affected in rats and cats by Cd feeding.

Testicular development. Cadmium feeding drastically affected testicle growth as indicated by measurements through the scrotum, and in weights on castration; also relative to body size they were smaller (table 4). With 160 ppm the testicles grew decidedly more slowly. (Any effect of 40 ppm was too small for detection by the methods used.) At 640 ppm there was very little testicle growth.

Previous work has shown that Cd injections greatly reduced testicle size in rats (20, 21) and goats (22). Parizek (20) observed that complete testicular necrosis was an important, specific, and quite constant sign of parenteral administration of Cd. However, no previous published work has been found concerning the effects of feeding Cd to any species upon testicles.

The mechanism responsible for the effect of Cd on gonads is mostly unknown. Parizek (20) suggested that Cd may act by a displacement of zinc from spermatogenic epithelium. Elcoate et al. (23) have demonstrated that inadequate uptake of zinc leads to degeneration of the sperma-

TABLE 4

Development of right testicle as measured by length times greatest diameter through the scrotum and weight at castration

	Treatment	Avg age, weeks					Avg final wt	% of body wt ($\times 10^{-3}$)
		4	8	12	16	20		
		cm	*cm*	*cm*	*cm*	*cm*	*g*	%
1	Control	3.76	8.12	10.03	16.88	22.95	35 a [1]	25.2
2	100 ppm Zn	4.62	7.88	9.36	16.20	22.35	40 a	27.9
3	40 ppm Cd	5.10	7.15	9.35	14.07	20.64	32 a	24.5
4	40 ppm Cd + 100 ppm Zn	4.70	7.52	10.15	15.50	22.54	34 a	24.2
5	160 ppm Cd	5.74	8.06	8.92	14.42	18.27	24 b	19.5
6	160 ppm Cd + 100 ppm Zn	5.17	6.94	9.05	13.22	19.83	29 ab	21.5
7	640 ppm Cd	5.45	8.65	7.64	6.58 [2]	7.99 [2]	7 [2]	11.1
8	2560 ppm Cd	5.14	7.44	6.08 [3]	6.77 [4]		— [5]	9.4

[1] Values not followed by the same letter are significantly different ($P = 0.05$) (16).
[2] Three surviving animals measured.
[3] Two surviving animals measured.
[4] One surviving animal measured.
[5] Testicles removed after death; average weight was 6 g.

togenic tubules, indicating the important role of this metal in spermatogenesis. Furthermore, the close physiochemical similarities between zinc and Cd make such a competitive antagonism between them appear likely. It is possible that the testicular necrosis results as a consequence of vascular injury to the testis (24).

Recovery from toxicity. Following termination of the treatment period, one calf that had received 640 ppm Cd was fed a practical-type diet. Improvement in appearance, feed consumption, growth, et cetera, were quite rapid. At 20 weeks of age it weighed 66 kg, at 28 weeks 120 kg, and at 12 months 282 kg. Average daily feed consumption for the last week it was fed Cd was 1.9 kg. Two weeks later the value was 3.1 kg. At 12 months of age semen was collected with the artificial vagina and no sperm were noted. At 13 months of age the one remaining testicle (the other was removed at 20 weeks) was removed. Substantial numbers of sperm were observed in the epididymis. However, most of these were abnormal. It was estimated that over 70% of the sperm had some motility which was almost 100% oscillatory with little or no progressive motility; about 90% were abnormal with more than 80% exhibiting bent midpieces or curled tails, or both; about 60% exhibited cytoplasmic droplets attached to the caudal one-third of the tail; and about 10% of the mobile sperm were headless. The testicle, trimmed to a kidney shape, weighed 317 g. Microscopic examination of the testicle tissue indicated that there was a wide variation in development and maturation. Many of the tubules showed evidence of spermatogenesis, whereas others were lined with only spermatogonia. The more mature tubules contained a few sperm cells. It appeared that the tissues were comparable to those of an individual approaching sexual maturity. Necropsy at 14 months of age indicated that this animal was in good condition but had liver and kidney scars.

These results indicate that a very severe and extended Cd toxicity may cause serious damage to the testicle development in the bovine. The sperm-producing tubules were not completely destroyed, and thus, it appears that either the effect of Cd on testicle tissue is not as drastic in the bovine as in the rat or that feeding does not give the same effect as injections. Clarification of this point will have to await further research.

Tissue concentrations of cadmium. Relatively little Cd was observed in tissues from a control calf and from one which had received 100 ppm zinc (table 5). Of the tissues analyzed, the liver contained the highest level. Literature reports of Cd values in tissues from animals including humans that have not been fed supplemental Cd are somewhat variable (5, 25, 26). It appears that age may be an important factor (26).

With the exception of skin and blood, the Cd content of all analyzed tissues was increased when 640 or 2560 ppm Cd were added to the diet (tables 5 and 6). The kidneys retained slightly more Cd per gram of dry tissue than the liver when either of these levels was fed. Both livers and kidneys of those receiving 2560 ppm tended to contain less Cd than the same organs from calves fed the 640 ppm diet.

Incisors of calves fed 2560 ppm Cd contained several times as much Cd as those given 640 ppm (table 5). The data do not reveal a ready explanation for this large difference. The average total intakes of Cd were not greatly different for the calves fed the 2560- and the 640-ppm Cd diets. No previous research data have been found concerning the Cd content of teeth of either normal or Cd-fed animals. It has been noted that ingested Cd tended to diminish the degree of pigmentation of rat incisor enamel (2, 3, 5). It was suggested that the effect might be related to ability of the Cd to interact with iron-containing proteins (3).

Effect of supplemental zinc on cadmium toxicity. The addition of 100 ppm zinc to diets containing 40 and 160 ppm Cd tended to increase feed consumption, weight gains, testicle size, hemoglobin, and blood zinc values (table 2). However, most of the differences did not reach statistical significance at the 0.05 probability level.

Supplee (17) reported that the addition of zinc to a turkey poult diet containing Cd prevented the appearance of most Cd toxicity symptoms. Others have shown that the destructive effect of subcutane-

TABLE 5

Cadmium and zinc content of various tissues

		Treatment groups			
		Control	100 ppm Zn	640 ppm Cd	2560 ppm Cd
No. of animals		1	1	3	4
Total Cd consumed, g				47(20–81) [1]	24(14–39) [2]
Body wt at end of treatment or death, kg		162	135	48	55
		ppm on dry basis		*ppm on dry basis*	*ppm on dry basis*
Incisor 1	Cd	0.7	0.7	22(9–41)	118(62–165)
	Zn	— [3]	— [3]	51(46–57)	72(61–87)
Incisor 2	Cd	1	1	31(13–54)	197(159–245)
	Zn	91	95	65(64–65)	71(66–79)
Liver	Cd	4	5	511(137–1023)	455(116–858)
	Zn	88	150	169(125–254)	166(135–211)
Spleen	Cd	< 1	< 1	25(11–39)	39(9–62)
	Zn	57	56	57(54–64)	64(54–69)
Kidney	Cd	< 2	< 2	672(479–1035)	501(146–718)
	Zn	59	84	110(81–147)	125(103–163)
Femur	Cd	0.8	< 0.4	3(2–5)	2(1–4)
	Zn	48	116	58(50–69)	91(30–170)
Hair	Cd	0.5	0.3	10(9–11)	10(9–13)
	Zn	92	76	119(113–126)	86(47–114)
Skin	Cd [4]	< 1.6	< 1.5	< 1.7	< 1.5
	Zn	10	12	16(15–18)	12(11–15)
		μg/ml	*μg/ml*	*μg/ml*	*μg/ml*
Blood	Cd [4]	< 0.05	< 0.05	< 0.05	< 0.10
	Zn	2.13	2.83	1.35(1.06–1.55)	2.30(1.55–2.68)

[1] Numbers in parentheses indicate range.
[2] Only 3 animals averaged as there was some indication that one calf's apparent consumption of 128 g Cd was in error due to his ability to separate his feed in such a way as to reduce the Cd intake.
[3] Samples lost during analysis.
[4] These values represent the lower limits of sensitivity of the analytical procedure for the sizes of samples and dilutions used. Thus, it can be said only that the values are below the amounts indicated. Other values preceded by < have the same meaning.

TABLE 6

Average zinc and cadmium content of testicles of calves receiving each treatment

Treatment	No. of calves	Zinc	Cadmium
		ppm on dry basis	
Control	4	65	3
100 ppm Zn	4	67	3
40 ppm Cd	4	65	3
40 ppm Cd + 100 ppm Zn	4	65	3
160 ppm Cd	4	62	4
160 ppm Cd + 100 ppm Zn	4	69	5
640 ppm Cd	4	61	14 *
2560 ppm Cd	4	65	16 *

* Significantly different from others ($P = 0.05$) (16).

ously administered Cd on the testis can be prevented by the simultaneous administration of large doses of zinc (21, 27, 28). Hill et al. (18) have reported that there is a copper component of Cd toxicity in addition to the zinc component. Their work with chicks suggested that Cd replaces both copper and zinc at active metabolic sites, probably enzymatic, thereby rendering these sites inactive. Thus, in the presence of Cd, added dietary copper and zinc were required to restore the animal to the nutritional status of controls. The addition of zinc reversed Cd-induced growth

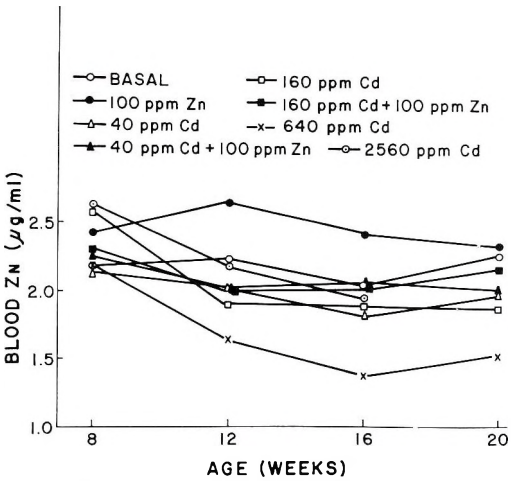

Fig. 6 Average whole blood Zn of calves fed different levels of Zn and Cd (unadjusted).

depression, whereas the addition of copper reduced the mortality (18).

Some of the clinical symptoms produced by the feeding of 2560- and 640-ppm levels of Cd resembled zinc deficiency symptoms quite closely (13). The calves fed 640 ppm Cd exhibited the most severe symptoms and were diagnosed on clinical examination as being zinc-deficient. Histopathological examination of skin from the neck and scrotum indicated a definite increase in the stratum corneum and a marked retention of nuclei. These changes are considered to be characteristic of parakeratosis (29).

Calves fed either 160 or 640 ppm Cd had a lower blood zinc content than controls ($P < 0.05$) (table 2 and fig. 6). Feeding 2560 ppm Cd did not lower blood zinc below that of controls. Those given the diets containing 640 and 2560 ppm Cd had higher zinc concentrations in both livers and kidneys than those fed the basal diet plus 100 ppm zinc (table 5). This is in agreement with work in rats indicating that Cd results in increased retention of radioactive zinc in these tissues (30). The other tissues analyzed were either approximately the same in zinc content or slightly lower (table 5). It may be that the zinc content in these organs increases because the Cd acts as an antimetabolite making certain enzyme molecules inactive. Thus a metabolic deficiency of zinc may exist in the presence of normal amounts due to the competitive action of the Cd. It is interesting to note that the zinc content of the liver and femur of the calf fed 100 ppm zinc increased twofold compared with the same tissues of the control.

It appeared that the feeding of Cd interfered with the utilization of dietary zinc, as the basal diet contained 27.4 ppm zinc. The apparent zinc deficiency that was produced might have been the result of decreased zinc absorption or increased zinc excretion. The increased levels of zinc in some tissues suggest that the competitive effects are at the tissue or cellular level. However, the higher levels of Cd drastically reduced feed intake. Thus some of the effects of the Cd may be caused by lower nutrient intake. Accordingly, these data do not always make possible a separation of those effects which are the direct result of Cd toxicity from those that are not. This could apply to several measures.

When interpreted in the light of previous work, it appears that the supplemental zinc in this experiment had a definite tendency toward reducing the toxic effects of the Cd. It also appears that the level of Cd in the diet tolerated is much higher for cattle than for the monogastric species previously studied. Thus it appears that future experiments should be conducted in which levels of Cd above 160 ppm are combined with supplemental zinc. Also, higher levels of zinc might be more effective.

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Investigation of Dietary Factors in Purified Diets for Ruminants [1,2]

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ABSTRACT The effect of supplements of vitamins other than A and D, 5% alfalfa and substitution of urea for casein in a purified diet developed for lambs was investigated. The complete diet contained casein, all the known vitamins, starch, glucose, fat, minerals and bicarbonates of sodium and potassium. Deletion of the vitamins other than A and D resulted in reduced gains. Addition of alfalfa had little or no effect on gains of lambs receiving casein diets. In the diets where urea was substituted for casein, alfalfa had a significant positive effect on gains of lambs. Samples of rumen fluid from lambs fed the urea-substituted diet without alfalfa had a significantly higher percentage of propionate and lower percentage of butyrate than the samples of rumen fluid taken from lambs fed the urea-alfalfa diet.

In previous work (1–3) a roughage-free purified diet was developed for lambs, which, except for supplements of sodium and potassium bicarbonates, was similar in most respects to purified diets utilized by small laboratory animals. The diet developed contained all the known vitamins, starch, glucose, fat, minerals, casein and the bicarbonates. In this early phase of the investigation emphasis was given to the utilization of the energy substrates of the diet. The subject of this report is a study designed to determine the effects of 3 other variables. Since vitamins had been added in the original diet without respect to need, attention was directed to whether the inclusion of vitamins other than A and D was necessary. A second variable was whether alfalfa contained additional factors not present in the purified diet developed, and a third was concerned with substitution of urea nitrogen for the preformed protein in the diet.

EXPERIMENTAL

The details of the biological procedures were similar to those described previously (1, 2). Lambs 2 to 3 weeks of age were separated from their dams and placed on a regimen of cows' milk administered by nippled bottles. At approximately 2.5 to 3 months of age the animals were divided into groups of 6 based on weight. The animals in each weight group were then assigned at random to the 6 experimental diets (table 1) to form a replication. Four replications of these diets were conducted over a period of 35 weeks in 1961 and 2 additional replications, during a period of 25 weeks in 1962. A total of 36 lambs was used in the study. All experimental animals received vitamin A and D capsules (table 1).

Except for the inclusion of 3% alpha-cellulose, diet 17 in table 1 was similar to the roughage-free purified diet developed previously (2, 3). This addition of cellulose to the diet reduced the stickiness of the feces, thereby facilitating cleaning of the cages. Diet 23 was similar to 17 except that the vitamin supplement (table 1) was omitted. Diet 24 contained 5% alfalfa without the vitamin supplement, whereas diet 25 contained vitamins and alfalfa. Glucose was reduced where alfalfa was added. Diets 26 and 27 contained urea nitrogen; diet 26 contained vitamins, and 27 contained alfalfa without the vitamins. Since the 5% alfalfa added to diet 27 supplied 1% protein, 2% casein was added to diet 26. The animals were housed in individual cages made of aluminum with aluminum diamond mesh flooring (see fig. 1). They were fed individually twice daily as much as they would consume. The diets were mixed

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TABLE 1

Composition of experimental diets

	Diet no.					
	23	17	24	25	26	27
	%	%	%	%	%	%
Casein	20	20	20	20	2.0	—
Urea	—	—	—	—	4.6	4.6
Glucose [1]	34.2	29.2	29.2	24.2	35.0	37.0
Starch	23.0	23.0	23.0	23.0	30.6	30.6
Vegetable fat [2]	4.0	4.0	4.0	4.0	4.0	4.0
Cellulose	3.0	3.0	3.0	3.0	3.0	3.0
Alfalfa leaf meal [3]	—	—	5.0	5.0	—	5.0
$KHCO_3$	4.0	4.0	4.0	4.0	4.0	4.0
$NaHCO_3$	6.0	6.0	6.0	6.0	6.0	6.0
$CaCO_3$	1.0	1.0	1.0	1.0	1.0	1.0
$CaHPO_4$	1.8	1.8	1.8	1.8	1.8	1.8
Vitamin mixture [4]	— [5]	5.0 [5]	— [5]	5.0 [5]	5.0 [5]	— [5]
Mineral mixture [6]	3.0	3.0	3.0	3.0	3.0	3.0

[1] Crystalline glucose, Corn Products Sales Company, Norfolk, Virginia.

[2] Grateful acknowledgment is made to Procter and Gamble Company, Cincinnati, Ohio, for Primex B and C (pure vegetable shortening).

[3] Alfalfa leaf meal (20% protein) was generously contributed by the National Alfalfa Dehydrating and Milling Company, Kansas City, Missouri.

[4] Vitamin mixture contained: (per 45.36 kg diet): thiamine·HCl, 400 mg; riboflavin, 850 mg; nicotinic acid, 1.13 g; Ca pantothenate, 1.42 g; pyridoxine·HCl, 570 mg; folic acid, 57 mg; *p*-aminobenzoic acid, 1.13 g; inositol, 11.35 g; biotin, 11.4 mg; choline chloride, 113.45 g; menadione (2-methyl-naphthoquinone), 115 mg; 0.1% vitamin B_{12} (with mannitol), 4.66 g; α-tocopheryl acetate, 570 mg; and glucose, 2132 g. Acknowledgment is gratefully made to Hoffmann-La Roche, Inc., Nutley N. J., for the biotin and to Merck and Co., Rahway, N. J., for contributing all other vitamins.

[5] Administered 4000 IU of vitamin A and 400 IU of vitamin D/day/45.36 kg body weight by capsules generously contributed by R. P. Scherer Corporation, Detroit, Michigan.

[6] Mineral mixture contained: (per 45.36 kg diet) KCl, 273 g; NaCl, 239 g; $MgSO_4$, 204 g; $CuSO_4 \cdot 5H_2O$, 893 mg; $FeSO_4 \cdot 2H_2O$, 7648 mg; $MnSO_4 \cdot H_2O$, 1399 mg; ZnO, 2263 mg; $CoCO_3$, 9 mg; KI, 6 mg; glucose, 633 g.

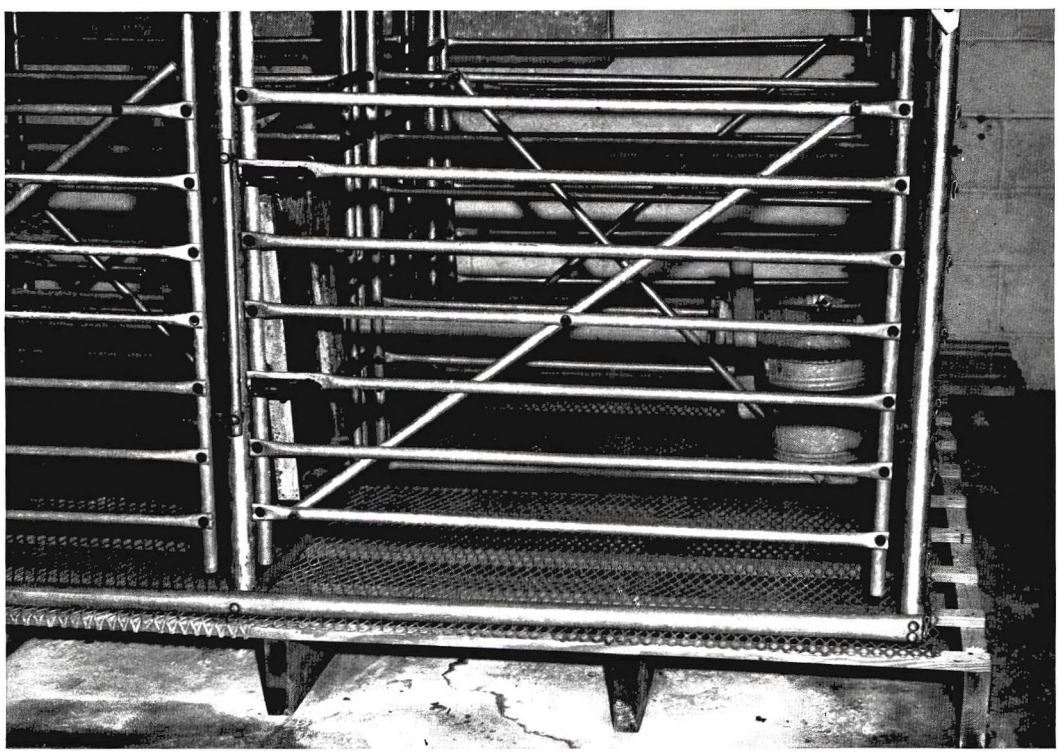

Fig. 1 Cages used for housing experimental lambs. Aluminum diamond mesh flooring and aluminum tubular siding.

twice weekly in a food mixer.[3] Records of daily feed intakes and weekly weights were kept during the experiment. Blood samples were taken at 4-week intervals and analyzed for calcium, phosphorous and magnesium as described previously (1). Sodium and potassium of the serum were determined with a flame photometer.[4] At the end of the experimental period samples of rumen fluid were taken from animals in 2 replications according to the following time sequence: a zero-hour sample was taken in the morning before forced feeding of 227 g of the diet (by stomach tube, in 1 liter of water) and subsequent samples were taken at 0.75, 1.5, 3, 6 and 8 hours thereafter. Volatile fatty acid analyses of the samples of rumen fluid were made by gas chromatography[5] according to the procedure of Erwin et al. (4). The results are reported as percentage of total volatile fatty acids. The value of a specific acid was arrived at by dividing the area under the chromatogram of the specific acid by the sum of the areas of all the acids of interest and multiplying the fraction by 100. Immediately after rumen samples were collected, pH was determined.[6] Sodium and potassium were determined by the flame photometer on the supernatant solution of rumen fluid samples spun in the centrifuge at 10,000 $\times$ g.

All data were subjected to analysis of variance: the 0.05 level of significance represents odds of 1:19 and the 0.01 level represents odds of 1:99.

RESULTS AND DISCUSSION

The mean values of gains, feed intake, feed efficiency and daily gains are shown in table 2. Alfalfa had little effect among the diets containing casein (diets 23, 17, 24 and 25), i.e., a mean gain of 27.9 kg was obtained for the lambs fed diets 23 and 17, without alfalfa, versus a mean gain of 28.6 kg for the lambs fed diets 24 and 25, which contained 5% alfalfa. The addition of vitamins, however, resulted in a significant effect ($P \leq 0.05$) on gains; the mean gain of the animals fed the vitamin-supplemented diets 17 and 25 was 31.3 kg as compared with 25.2 kg for the lambs fed diets 23 and 24 without vitamins. The effect of adding B-vitamins to ruminants' diets has generally been reported to be negative (5–8). The positive results obtained in this study are most probably the result of the inadequate synthesis of B-vitamins by the prevailing flora. This effect could have been due to the young age of the lambs at the start of the experiment, the dietary pre-experimental regimen, or to the lack of roughage in the purified diets.

In the comparison between the 2 diets containing urea, the inclusion of alfalfa significantly improved the gains of lambs. Lambs fed the casein diet 17, which was supplemented with vitamins, gained more than those fed the urea diet 27 containing alfalfa without added vitamins. Lambs fed diet 27, however, made gains comparable with those fed the casein diet 23, unsupplemented with vitamins.

It is unlikely that the difference between diets 27 and 26, with and without 5%

[3] Hobart Manufacturing Company, Troy, Ohio.
[4] Baird Atomic Flame Photometer Model Ky, Baird Atomic, Inc., Cambridge, Massachusetts.
[5] Perkin-Elmer Vapor Fractometer Model 154 (modified for flame ionization operation), Perkin-Elmer Corporation, Norwalk, Connecticut.
[6] Beckman Zeromatic pH Meter, Beckman Instruments, Inc., Fullerton, California.

TABLE 2

Effect of vitamins, source of dietary nitrogen and alfalfa on gains of lambs fed purified diets

Criteria [2]	Diet no. [1]					
	23	17	24	25	26	27
Mean gain (kg)/animal	24.7 [3]	31.0	25.6	31.6	15.1	23.8
Mean feed intake (kg)/animal	202.0	213.0	210.0	234.0	186.0	206.0
Kilograms intake/kg gain	8.2	6.9	8.2	7.4	12.4	8.6
Average daily gain (kg)	0.111	0.141	0.115	0.142	0.068	0.107

[1] Diets 17, 25 and 26 contained a vitamin supplement; 23, 24 and 27 did not. Diets 23, 17, 24 and 25 contained casein; 26 and 27 contained urea. Diets 24, 25 and 27 contained 5% alfalfa; 23, 17 and 26 contained none.
[2] Mean of 6 animals/diet. Average initial weight for lambs in replications 1, 2, 3, 4, 5 and 6 were 12.7, 13.2, 19.1, 13.6, 14.1 and 15.0 kg, respectively.
[3] SE of mean, 2.67 kg.

alfalfa, is due to differences in methionine, which has been shown to improve urea diets (9). Calculations made from published values of the methionine content of casein (10) and alfalfa (11) show that the 2% casein in diet 26 carried more methionine and more total sulfur amino acids than the 5% alfalfa.

Blood analyses. The calcium, phosphorus, magnesium, sodium and potassium values of the serum of the experimental animals (table 3), are means of samples taken at 4-week intervals during the experimental period. The lambs fed the urea diet 26 had a slightly lower serum phosphorus and higher serum calcium than animals receiving the other diets. Serum phosphorus trends for all the animals on the experiment did not appear to decrease as much as one would expect in comparison with lambs of comparable age fed a hay diet (12). Marked differences among diets for any of the serum constituents measured were not obtained. Serum sodium and potassium were within the normal range for lambs despite the high levels of sodium and potassium in the diet.

Rumen fluid analyses. The volatile fatty acid results are presented in figure 2. Significant differences ($P \leq 0.01$) in percentage of propionate and butyrate were obtained in the contrast between the urea

TABLE 3

Mineral analyses of serum [1] of experimental animals

	Diet no.					
	23	17	24	25	26	27
Calcium, mg/100 ml serum	12.38	12.86	12.10	12.46	13.07	12.98
Phosphorus, mg/100 ml serum	9.42	9.59	9.42	9.53	8.66	9.39
Magnesium, mg/100 ml serum	2.38	2.37	2.51	2.43	2.42	2.41
Sodium, mEq/liter serum	160.0	162.0	161.0	161.0	161.0	164.0
Potassium, mEq/liter serum	6.7	6.9	6.6	7.0	6.6	7.2

[1] Mean of 6 animals/diet, 6 samples/animal (samples taken monthly).

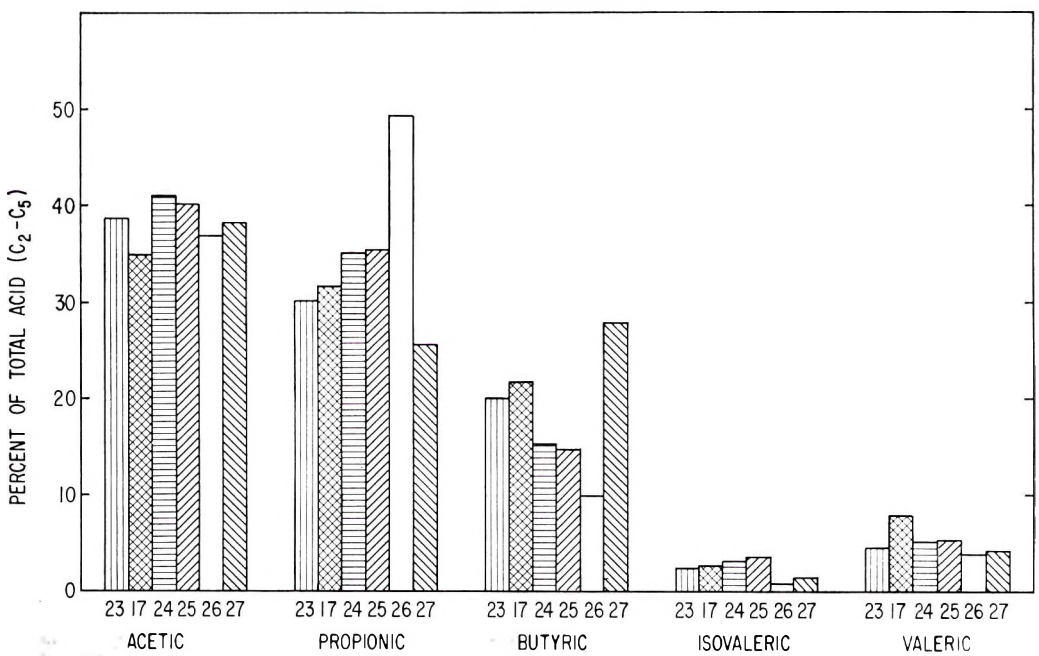

Fig. 2 Volatile fatty acids in samples of rumen fluid of lambs fed the experimental diets 23, 17, 24, 25, 26 and 27. Each bar is the average of 12 rumen samples, six from each of 2 animals, taken before feeding and at 0.75, 1.5, 3, 6 and 8 hours after feeding.

diets 26 and 27. The percentage of propionic acid obtained for diet 26 was 49.3, whereas for diet 27 it was 25.6; the values obtained for butyric acid fell in the reverse order, 9.9 and 28.0%, respectively. Thus, the addition of 5% alfalfa to the urea diet lowered the propionate and raised the butyrate to the degree that the values fall more in line with those obtained with the other diets. Since ruminal butyrate of sheep fed purified diets has been shown to arise from acetate (13), it is noteworthy that the butyrate percentages were changed without a concomitant change in acetate. Whether the change in propionate and butyrate associated with the inclusion of alfalfa in the urea diets is the consequence of a change in rumen flora or from co-factors supplied by the alfalfa cannot be determined from the data of this study.

Marked differences in the average pH value and mean concentration of sodium and potassium of the rumen samples taken in the sequential time study at the end of the growth trial were not obtained. The average pH was 6.27 ± 0.677; the mean sodium concentration was 85.2 ± 19.6 mEq/liter and that for potassium was 82.0 ± 18.2. The potassium concentration, however, was higher in relation to that of sodium in the rumen fluid than ratios reported in the literature (14, 15). The sodium-to-potassium ratios, moreover, remained fairly uniform throughout the 8-hour experiment.

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Vitamin B_6 Depletion in Man: Urinary Taurine and Sulfate Excretion and Nitrogen Balance [1]

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ABSTRACT Six young men were fed a partially purified diet which supplied 0.16 mg of vitamin B_6 daily. When biochemical changes became apparent, the diet of each subject was supplemented with pyridoxine (0.6 or 0.9 mg). No clinical symptoms were noted which could be attributed to the vitamin B_6 depletion. The diet which was effective in producing the depletion was well accepted by the subjects and was adequate in all respects except for vitamin B_6; it contained 100 g of protein and was composed of casein, gelatin, rice, sucrose, jelly, carbonated beverages, selected fruits and vegetables low in vitamin B_6, a fat mixture which supplied 40% of the calories, L-methionine, and mineral and vitamin supplements. A 3.4-g loading dose of L-cysteine was administered orally several times during the study. At the height of vitamin B_6 depletion only one subject reflected the loading dose with an appreciably increased excretion of taurine, whereas, all subjects excreted increased amounts of taurine after the cysteine loading dose when their diets had been supplemented with pyridoxine. Urinary sulfate excretion after the cysteine loading dose and the nitrogen balance during the study were not affected by vitamin B_6 depletion or repletion.

Several investigators have attempted to study qualitative or quantitative aspects of the vitamin B_6 requirement of adult man by feeding diets which contained small amounts of the vitamin and by measuring the changes that occurred in one or more bichemical parameters. Urinary xanthurenic acid excretion after a loading dose of tryptophan has been the biochemical measurement most frequently made to assess vitamin B_6 nutriture (1–5),[3] and it has generally been found that a low intake of the vitamin results in an increased xanthurenic acid excretion. Low vitamin B_6 intakes have been reported to result in other biochemical and physiological changes in the adult human; among these are decreases in blood pressure (6), hemoglobin concentration (2, 6), hematocrits and red blood cell counts (6), lymphocyte counts (5), serum glutamic-oxalacetic transaminase (4), and changes in electroencepalographic recordings.[4]

Essentially 2 types of low vitamin B_6 diets have been fed. Purified diets using casein as a protein source and containing less than 100μg of vitamin B_6 daily have been fed by some investigators (1, 2, 6),[3,4] and natural food diets containing approximately 400 to 600μg or more of vitamin B_6 per day have been fed by others (3–5). The feeding of a completely purified diet to human subjects presents palatability problems, whereas the feeding of a natural food diet hinders the development of clear-cut biochemical abnormalities within a reasonable length of time because vitamin B_6 occurs in significant amounts in most foods and particularly in protein-rich foods.

Comparisons among previous studies are difficult because of differences in the composition of the diets fed and in the biochemical parameters measured. Little attempt has been made to measure many different parameters in the same subjects during a low vitamin B_6 intake and to determine which is most sensitive to vitamin B_6 depletion. Although xanthurenic acid excretion after a tryptophan loading dose has been the criterion most frequently used to demonstrate a depletion of vitamin B_6, it is possible that the measurement of other

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biochemical parameters might be more sensitive. It has been suggested (7) that the changes in sulfur amino acid metabolism might provide more sensitive criteria than those previously used since some of the enzymes responsible for the metabolism of these amino acids are extremely sensitive to vitamin B_6 depletion in the rat.

The present report describes a partially purified diet which contains less than 160 μg of vitamin B_6 daily and which is palatable enough to be consumed for prolonged periods of time. Feeding this diet to 6 men made possible the simultaneous study of many biochemical parameters in the same subjects. Results of the following analyses will be reported in this paper: urinary taurine and sulfate excretion before and after a cysteine loading dose; nitrogen balance; and urinary excretion of ammonia, urea, uric acid, and α-amino nitrogen. Subsequent papers will report results of other analyses including the following: urinary excretion of tryptophan and niacin metabolites; activity of serum glutamic-oxalacetic and serum glutamic-pyruvic transaminases; serum cholesterol concentration; blood and urinary levels of vitamin B_6; blood concentrations of pyridoxal phosphate; and 4-pyridoxic acid excretion.

EXPERIMENTAL

Diet. The high-protein, low-vitamin B_6 diet developed in this laboratory for the purpose of studying the adult requirement for vitamin B_6 is shown in table 1. Rice and selected fruits and vegetables low in vitamin B_6 were the natural foods included in the diet. The total nitrogen content of the daily diet was 16.6 g; vitamin-free casein supplied 10.0 and gelatin, 5.0 g of nitrogen. This moderately high intake of protein was chosen in preference to a lower one since high intakes of protein have been shown to increase the vitamin B_6 requirement of rats (8, 9). It has been demonstrated that methionine in particular increases the vitamin B_6 requirement of

TABLE 1

Composition of high-protein, low-vitamin B_6 diet

		Vitamin B_6 content	
		per gram	per day
	g	μg	μg
Casein [1]	70.1	0.099	6.94
Gelatin [2]	30.7	0.091	2.79
Rice	20.0	0.824	16.48
Rice cereal [3]	45.0	0.824	37.08
Peaches	100.0		
Pears	100.0	0.200	64.00
Grapefruit juice	120.0		
Carrots	15.0		
Celery	15.0	0.620	27.90
Onions	15.0		
Sucrose	90.0		
Fat mixture [4]	85.0		
Butter oil	27.0		
Vitamin mixture [5]	one capsule		
Mineral mixture [6]	3.6		
Methionine	1.665		
Ascorbic acid	0.050		
Folic acid	0.0001		
Total			155.2

[1] Sheffield Chemical Company, Inc., Norwich, New Jersey.
[2] Nutritional Biochemicals Corporation, Cleveland.
[3] Cream of Rice, Grocery Store Products Company, West Chester, Pennsylvania.
[4] The fat mixture consisted of the following: (in g/100 g) beef tallow, 22.1; hydrogenated vegetable shortening, 25.6; lard, 40.9; cottonseed oil, 11.4.
[5] A gift from Hoffmann-LaRoche, Inc., Nutley, New Jersey; the vitamin mixture was given in a capsule at breakfast and consisted of the following: (in milligrams) thiamine·HCl, 3; riboflavin, 3; D-pantothenyl alcohol (D-Panthenol), 4.5; niacinamide, 9; D-biotin, 0.15; cyanocobalamin, 0.001; *dl*-α-tocopheryl acetate, 4.5; and 4500 USP units of vitamin A.
[6] The mineral mixture of Leverton (12) was used and supplied the following: (in milligrams) calcium, 549; phosphorus, 300; magnesium, 200; iron, 16; copper, 2; iodide, 0.15; manganese, 2; and zinc, 0.9.

rats (10); therefore, capsules containing L-methionine were fed with each meal. This addition of 1.66 g of L-methionine daily increased the total sulfur-containing amino acids to 4.8 g/day, an amount which is less, however, than would have been contributed by 100 g of egg protein.

Butter oil and a mixture of fat supplied 40% of the calories in the diet. The composition of the fat in the diet was similar to that of the fat in the average American diet (11); it was calculated that approximately 10% of the fat was linoleic acid. Carbonated beverages or jelly were added in amounts to bring the daily caloric intake up to 2500 kcal. When a subject required more than 2500 kcal, he was fed candy made from the fat mixture, butter oil, sucrose, and water; the fat mixture and butter oil supplied 40% of the calories in the candy.

With the exception of vitamin B_6, adequate amounts of all vitamins known to be essential for man were supplied daily. Approximately one-third of the mineral mixture described in table 1 was fed at each meal. The various components of the diet were analyzed for vitamin B_6 in the laboratory of the Wisconsin Alumni Research Foundation using *Saccharomyces carlsbergensis* as the test organism. On the basis of the results of these analyses (table 1) the total vitamin B_6 content of the daily diet was calculated to be 155 μg.

Rice cereal served as a carrier for the butter oil, fat mixture, and casein. Individual portions of casein and the fat were blended in small casserole dishes and the cooked rice cereal was added. Carrots, onions, celery, rice, and the remainder of the fat mixture were made into a casserole. Individual daily portions of gelatin were mixed with sucrose and a powdered drink concentrate and boiling water was added in an amount to make a gel of an acceptable consistency. One-third of the gelatin and casein was fed at each meal. For additional bulk, subjects were given methylcellulose to take at their own discretion. The meals were served at regularly scheduled times in the metabolic unit and the preparation of the meal was under the supervision of a nutritionist.

Subjects. The 6 subjects who were fed this diet were men ranging in age from 23 to 35 years and, on the basis of a physician's examination prior to the study, all were considered to be in good health. All were students who engaged in their normal activities during the course of the study. Descriptive data concerning the subjects are presented in table 2. At each evening meal the subjects filled out a short questionnaire which allowed them to indicate any unusual physical activities during the day as well as any changes in emotional attitudes. They were examined by a physician at the beginning and end of the study and on days 26, 34, and 42.

Procedure. During days 1 through 5 of the study the subjects consumed self-chosen diets; during days 6 through 55 the subjects were fed the low vitamin B_6 diet described above. A 5.0-g L-cysteine monohydrochloride monohydrate loading dose 3.4g or 28.47 mmoles of cysteine) was given on selected days throughout the study (table 3, line 2). Urinary taurine and sulfate excretion were measured on the day prior to and the day of the loading dose. Sulfate excretion was measured for an additional day after the loading dose. Urinary urea, uric acid, α-amino nitrogen, and ammonia were measured before and after each cysteine and tryptophan loading dose and on certain other days during the

TABLE 2

Vital statistics and caloric intake of each subject

Subject	Age	Weight		Height	Calories/ day
		Initial	Final		
	years	*kg*	*kg*	*cm*	*kcal*
1	26	69.4	67.1	178	2500
2	25	81.6	79.6	183	3100
3	24	77.8	75.5	180	3300
4	35	69.4	66.5	178	2500
5	23	61.0	58.7	168	3000
6	30	67.1	66.7	173	2700

TABLE 3

Experimental design showing days of study on which amino acid loading doses and pyridoxine supplementations were given and days on which blood samples were taken

Treatment	Days of study
Tryptophan load dose [1]	1, 10, 20, 30, 40, 45, 51, 54
Cysteine load dose [2]	4, 13, 23, 31, 41, 49, 52, 55
Venipuncture [3]	1, 6, 11, 16, 21, 26, 31, 36, 41, 46, 51
Fingerprick [4]	1, 2, 7, 10, 15, 17, 20, 25, 27, 30, 35, 37, 40, 45, 47, 50, 55
0.6 mg pyridoxine supplement to subjects 4 and 5 [5]	33 through 52
0.9 mg pyridoxine supplement to subjects 2 and 6	42 through 52
0.6 mg pyridoxine supplement to subjects 1 and 3	46 through 52
50 mg pyridoxine supplement to all subjects [6]	53 through 55

[1] Two grams of L-tryptophan administered in tablet form at breakfast.
[2] Five grams of L-cysteine-monohydrochloride-monohydrate administered in capsules at breakfast.
[3] Twenty cubic centimeters of blood were removed from the arm every 10 days to be used for hemoglobin, transaminase, and pyridoxal phosphate determinations. Ten cubic centimeters were removed 5 days later for vitamin B_6 analysis.
[4] One fingerprick every 10 days was used for a complete cell count; the others were used for measurement of free and total blood cholsterol.
[5] Given as a pyridoxine hydrochloride solution in 0.02 N acetic acid added to the fruit juice at breakfast.
[6] Given as pyridoxine hydrochloride in capsule at breakfast.

study. The days on which other biochemical measurements were made are shown in table 3. All fecal collections were frozen immediately for future nitrogen analysis. Urine was collected under toluene and refrigerated until the analyses were completed, usually within 48 hours. The completeness of each collection was checked by creatinine analysis.

Marginal supplementation with pyridoxine was begun when abnormalities in tryptophan metabolism became pronounced (13). Since the subjects did not become depleted at the same rate, all were not given the supplement at the same time. The times and amounts of supplementation are shown in table 3.

Methods. The analyses reported in this paper were made according to the following methods: urinary taurine in unhydrolyzed samples by the method of Pentz et al. (14); total urinary sulfate by the method of Folin (15); urinary and fecal nitrogen by the boric acid modification of the Kjeldahl method (16); urinary ammonia, corrected for α-amino nitrogen, by the formol titration method (17); urinary urea by the method of Marshall (18); urinary uric acid by the method of Benedict and Franke (19); and α-amino nitrogen by a modification of the method of Pope and Stevens (20). Probability values were calculated using Student's t test for paired observations.

RESULTS

Frequent examinations by a physician during the period of depletion failed to reveal any clinical symptoms attributable to a low intake of vitamin B_6 in the 6 subjects fed the diet without supplementation for 27, 36, or 40 days. Headaches were reported by some of the subjects during the period in which they were depleted in vitamin B_6, but these same subjects reported headaches with approximately the same frequency during the time in which the diet was supplemented with the vitamin.

Taurine excretion before (basal level) and after each cysteine loading dose is shown in table 4. The basal level of tau-

TABLE 4

Effect of vitamin B_6 on the 24-hour urinary excretion of taurine by young men before and after a cysteine loading dose

Diet	Day of study	Cysteine dose administered [1]	Subject no.						Amount of cysteine dose excreted as taurine
			1	2	3	4	5	6	
			μmoles/24 hours						%
Self-selected diet	3	no	2165	2053	1055	975	1055	791	
	4	yes	3004	2373	1710	1502	1478	1638	1.1–3.0
Low vitamin B_6 diet	12	no	951	1183	1031	1318	791	951	
	13	yes	1111	1238	1238	1606	871	1031	0.2–1.0
Low vitamin B_6 diet (at height of depletion)	30 [2] or 40 [2]	no	847	1055	1111	999	791	847	
	31 or 41	yes	951	1080	1350	847	735	895	0.0–0.8
Low vitamin B_6 diet + 50 mg pyridoxine	54	no	791	1238	1294	711	639	767	
	55	yes	1135	1710	1742	999 [3]	999 [3]	1055	1.0–1.7

[1] Five grams of L-cysteine-monohydrochloride-monohydrate (28,470 μmoles cysteine) were administered orally on days 4, 13, 31, and 41.
[2] Experimental days 30 and 31 for subjects 4 and 5; days 40 and 41 for all other subjects. These were the days of the last loading dose before marginal pyridoxine supplementation was begun.
[3] Urine collections for subjects 4 and 5 were inadvertently pooled on day 55.

rine excretion was lower for four of the six subjects after 7 days of depletion (day 12) than it was on day 3 when the subjects were eating self-chosen diets. For four of the six subjects it continued to decrease throughout the study, whereas for subjects 2 and 3 it increased after the pyridoxine supplementation. On day 4 of the study when the subjects were eating a self-chosen diet the cysteine loading dose was reflected by an increased taurine excretion by all subjects, and the percentage of the cysteine dose accounted for as taurine ranged from 1.1% for subject 2 to 3.0% for subject 6. After the subjects had eaten the low vitamin B_6 diet for only 8 days, the percentage of the cysteine dose accounted for as taurine had decreased in all subjects and ranged from 0.2% for subject 2 to 1.0% for subject 4. At the time of the last cysteine load before pyridoxine supplementation (day 41 for subjects 1, 2, 3, and 6 and day 31 for subjects 4 and 5) the loading dose did not result in an appreciable increase in taurine excretion except for subject 3. However, on day 55 after the subjects had been receiving pyridoxine supplementation the percentage of the cysteine accounted for by an increase in taurine excretion ranged from 1.0% for subjects 4 and 6 to 1.7% for subject 2. The amount of taurine excreted in response to the cysteine dose on day 55 after vitamin B_6 repletion was significantly more than that excreted after 8 days of vitamin B_6 depletion ($P < 0.01$) and that excreted at the height of vitamin B_6 depletion ($P < 0.001$); however, the amount excreted on day 55 was lower than that excreted on day 4 before the subjects were depleted of vitamin B_6.

The increase in urinary sulfate excretion after the cysteine loading dose is shown in table 5. At the time of the last loading dose of cysteine prior to pyridoxine supplementation, the subjects excreted a greater part of the sulfur in the cysteine loading dose as total sulfate than they did on day 13 ($P < 0.01$). The part of the loading dose appearing as urinary sulfate after pyridoxine supplementation decreased for four of the subjects, but continued to increase for subjects 2 and 3.

The nitrogen balance for each subject during days 6 through 55 of the study is

TABLE 5

Effect of vitamin B_6 on the conversion of a cysteine loading dose to urinary sulfate [1]

Diet	Day of Study [2]	Subject no.					
		1	2	3	4	5	6
		mmoles/24 hr					
Self-selected diet	4	30.03	22.44	17.94	19.47	23.66	25.22
Low vitamin B_6 diet	13	19.28	19.84	21.47	24.94	22.62	21.62
Low vitamin B_6 diet height of depletion [3]	varied	23.19	24.06	23.75	25.81	27.91	26.56
Low vitamin B_6 diet + 50 mg pyridoxine	55	22.16	29.56	25.12	24.66[4]	25.06[4]	23.38

[1] Sulfate excreted for the 24 hours after the cysteine loading dose minus sulfate excreted for the 24 hours prior to the loading dose.
[2] Five grams of L-cysteine monohydrochloride monohydrate (28.47 mmoles cysteine) were administered orally at the beginning of each of these 24-hour periods.
[3] Experimental day 31 for subjects 4 and 5; day 41 for all other subjects. These were the days of the last loading dose before marginal pyridoxine supplementation.
[4] Urine collections for subjects 4 and 5 were inadvertently pooled on day 55.

shown in figure 1. There was no apparent effect of vitamin B_6 depletion on nitrogen retention in these subjects. Subjects 2 and 6 were in negative balance during part of the study. However, except for days 6 through 15, during which time the subjects were adjusting to the experimental diet, the other 4 subjects were in positive balance. Subject 5 stored large amounts of nitrogen throughout the study. The amounts of urea, uric acid, and α-amino nitrogen excreted varied somewhat from one day to the next for each subject but were not affected by the amino acid loading doses or by the state of vitamin B_6 nutriture. In terms of grams of nitrogen excreted per day, urea accounted for 10 to 14 g, uric acid for 0.15 to 0.35 g, and the α-amino nitrogen fraction for 0.25 to 0.50 g. Urinary ammonia, which ranged from 0.4 to 1.4 g of nitrogen/day, increased each time the cysteine loading dose was given; the increase ranged from 0.1 to 0.7 g of nitrogen daily. An increase in titratable acid in the urine has been reported after the feeding of large amounts of methionine and has been assumed to be due to hydrogen ions resulting from the conversion of cysteine to cysteinesulfinic acid and the conversion of β-sulfinylpyruvate to sulfate and pyruvate (21). The increase in ammonia excretion in the present study following the cysteine loading dose probably resulted from the oxidation of the neutral sulfur to sulfate.

DISCUSSION

Taurine can be formed through the decarboxylation of cysteinesulfinic acid, an oxidation product of cysteine, to yield hypotaurine which upon oxidation yields taurine. The activity of cysteinesulfinic acid decarboxylase disappears from the liver of rats deficient in vitamin B_6 (22, 23). There is evidence that the level of the apoenzyme is decreased. Part of the lost activity of the enzyme can be restored during the early stages of deficiency by addition of pyridoxal phosphate to liver homogenates in vitro (22). However, after rats have been fed the deficient diet for several days reactivation of the cysteinesulfinic acid decarboxylase in vitro is not possible (24). Thus, a loss of the apoenzyme in the absence of the co-factor is suggested. When pyridoxine is administered to vitamin B_6-deficient rats over a 6-hour period before the livers are removed, there is an increase in cysteinsulfinic acid decarboxylase activity. Since this increase can be inhibited by puromycin, control of the apoenzyme level by the co-factor is suggested.[5] In the present study, the ability to convert the cysteine loading dose to urinary taurine was decreased as the subjects became depleted in vitamin B_6 and was not completely restored by supplementation with pyridoxine.

[5] Greengard, O., and M. Gordon 1963 Increase in the apoenzyme levels of two pyridoxal phosphate-requiring liver enzymes by pyridoxine administration in vivo. Federation Proc., 22: 232 (abstract).

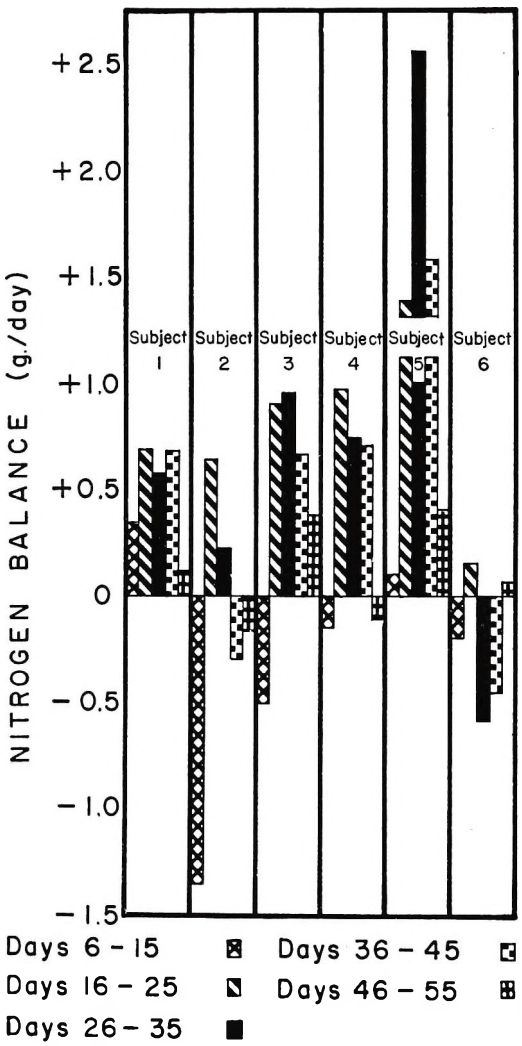

Fig. 1 Nitrogen balance of the 6 subjects who were fed the low vitamin B_6 diet. Although pyridoxine supplementation was begun at different times all subjects were receiving pyridoxine during days 46 through 55.

If vitamin B_6 depletion affects the apoenzyme level, it is possible that taurine excretion after the cysteine loading dose might have been increased further had a longer period of repletion with more than marginal amounts of vitamin B_6 been allowed.

The possibility of using a cysteine loading dose as a means of evaluating vitamin B_6 nutrition was considered by McAfee and Williams (25) who administered large doses of cysteine to rats. The adult rats that had been fed a commercial rat diet converted from 0.45 to 0.68% of the cysteine loading dose to taurine. Supplementation of the diet of these rats with 50 μg of pyridoxine hydrochloride daily for 2 weeks resulted in a two- to tenfold increase in the conversion of the cysteine loading dose to taurine. In the present study human subjects converted from 0.0 to 0.8% of the cysteine loading dose to taurine when depleted in vitamin B_6 and 1.0 to 1.7% after pyridoxine supplementation.

It is not known whether the decrease in the basal level of taurine excretion which occurred when subjects were fed the experimental diet was due to the low intake of vitamin B_6 or to other differences between the self-chosen and experimental diets. It appears that the total sulfur amino acid content of the self-chosen and experimental diets was similar since the urinary sulfate excretion was approximately the same with both diets. However, the self-chosen diets undoubtedly were higher in taurine than the experimental diet.

Even though the subjects did not excrete a significant part of the cysteine loading dose as urinary taurine at the height of vitamin B_6 depletion, they were still excreting appreciable basal amounts of taurine. In the rat 2 vitamin B_6-dependent pathways for the formation of taurine have been demonstrated; one is through the decarboxylation of cysteic acid to form taurine (26) and the second is through the decarboxylation of cysteinesulfinic acid to form hypotaurine (22) which is subsequently oxidized to taurine (27). It is assumed that the latter pathway is the most important since the rate of decarboxylation of cysteinesulfinic acid is faster than that of the decarboxylation of cysteic acid (23). The possibility that a third pathway exists for the formation of taurine has been suggested (28, 29). The substantial basal excretion of taurine in the present study when the subjects were depleted in vitamin B_6 may tend to lend support to this suggestion.

The nitrogen balance of the 6 subjects was not affected by vitamin B_6 depletion. Faber et al. (30) reported that a combined

pyridoxine and pantothenic acid deficiency caused a negative nitrogen balance in adult male subjects and assumed that this was due to the pyridoxine deficiency since a decreased nitrogen retention has been reported to result from a pyridoxine deficiency in the rat (31). It is possible, however, that the pantothenic acid deficiency may have influenced nitrogen balance in the subjects of Faber and his associates. The fact that nitrogen balance during the last 10 days of the present study tended to be less positive for most subjects may have been due to the 5 amino acid loading doses given during this period. It is known that large excesses of some amino acids create an imbalance and depress the utilization of other amino acids.

The subjects lost between 0.4 and 2.9 kg of body weight during the study. However, the caloric intake ranged from 36 to 49 kcal/kg of the body weight, per day. Whether the weight loss was due to caloric insufficiency or to other factors is not known.

The use of the diet described in this paper should facilitate the study of the quantitative requirement of vitamin B_6 for man and should prove useful in studying the various factors which affect the requirement since biochemical changes occur within a relatively short time. The depletion obtained using this diet for approximately 4 weeks is only moderate. Apparently, severe depletion of vitamin B_6 in the rat can result in a different biochemical picture from that observed with a moderate depletion. For example, cysteinesulfinic acid decarboxylase activity is retained in rat brain until the rat becomes severely depleted in the vitamin, whereas it is readily lost in rat liver (23), thus indicating that there may be preferential binding of pyridoxal phosphate by the brain enzyme. A similar observation has been reported with respect to hydroxytryptophan decarboxylase (32). In a moderate deficiency where skin lesions constitute the major symptom, the hydroxytryptophan decarboxylase activity in rat liver is markedly reduced but is unchanged in the brain. However, a severe depletion which results in convulsive seizures is characterized by a greatly lowered activity of hydroxytryptophan decarboxylase in brain. These observations suggest that a severe depletion of vitamin B_6 may be undesirable and unnecessary in attempts to characterize biochemical changes which occur in the human as a result of suboptimal vitamin B_6 intake.

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Vitamin B_6 Depletion in Man: Urinary Excretion of Tryptophan Metabolites [1]

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ABSTRACT The effects of vitamin B_6 depletion on the metabolism of tryptophan by 6 male subjects were studied during a 55-day experiment. The excretion of several urinary metabolites of the amino acid was measured quantitatively during a 5-day pre-depletion period when the subjects ate self-selected diets and at intervals throughout the study while they consumed a diet containing only 0.16 mg of vitamin B_6 daily. In response to the ingestion of a 2-g supplement of L-tryptophan, the levels of hydroxykynurenine, kynurenine, xanthurenic acid, acetylkynurenine and kynurenic acid were significantly increased in the urine of the subjects as the deficiency was induced. Two subjects showed evidence of abnormal tryptophan metabolism after only 5 days of depletion. The rate and extent to which the individuals exhibited abnormal tryptophan metabolism varied among the 6 males and determined the order in which supplementation with pyridoxine was begun. The excretion of metabolites following tryptophan loading decreased to the normal range when the subjects were given supplemental pyridoxine at a level of 0.6 or 0.9 mg/day. In three of the men, however, tryptophan metabolism did not return to pre-depletion status until the end of the experiment when 50 mg of pyridoxine were administered on 3 consecutive days. The pattern of abnormal tryptophan metabolism observed in this situation was strikingly similar to that found in tuberculosis patients receiving deoxypyridoxine and thus appears to indicate that the pyridoxine antagonist and a dietary deficiency of the vitamin prevent normal functioning of the vitamin B_6-dependent enzymes involved in tryptophan metabolism in an analogous manner.

In studying pyridoxine deficiency induced in adults by means of dietary management (1–4),[4] and by drug administration with or without dietary restrictions (5–8), the excretion of xanthurenic acid has been noted to increase with a deficiency of the vitamin. In most investigations, the excretion of other tryptophan metabolites has not been measured. Studies in this laboratory have determined the excretion of several tryptophan metabolites in the urine of patients with various clinical conditions and the influence of pyridoxine on tryptophan metabolism in certain conditions (9). From such studies, the value of measuring several urinary metabolites in differentiating specific biochemical abnormalities has been demonstrated. Thus, it has been shown that there was an abnormally high level of xanthurenic acid in the urine after the ingestion of 2 g of L-tryptophan by pregnant women (10) or by tuberculosis patients being treated with deoxypyridoxine (11). However, patients with scleroderma, porphyria, neoplastic diseases (9) or tuberculosis patients being treated with isoniazid (11) also had abnormal tryptophan metabolism, but with essentially normal levels of urinary xanthurenic acid after the ingestion of tryptophan.

Reported here are the results of a study of tryptophan metabolism in normal subjects given a diet deficient in vitamin B_6. These data are discussed in relation to the abnormal tryptophan metabolism found in patients with vitamin B_6 deficiencies produced by isoniazid and deoxypyridoxine.

EXPERIMENTAL

The 6 males who were the subjects in this experiment have been described in the

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[2] American Cancer Society — Charles S. Hayden Foundation Professor of Surgery in Cancer Research.

[3] Supported by Public Health Service Research Career Development Award no. CA-K3-18,404 from the National Institutes of Health.

[4] Baker, E. M., H. E. Sauberlich and J. E. Canham 1963 Vitamin B_6 requirement of the human. Federation Proc., 22: 322 (abstract).

preceding paper (12). The dietary treatment, biochemical measurements made, general information concerning the study and some of the results have also been presented previously (12). Pertinent facts dealing with the design of the experiment that have already been given will be repeated only when they will facilitate the presentation of the data from this aspect of the study.

The tryptophan metabolism of the 6 subjects was studied at intervals during the 55-day experiment. To have a base-line for comparison with data obtained later, the subjects were studied initially during the first 5 days of the experiment (this has been termed the pre-depletion period) when they were consuming self-selected diets. The subjects collected a 24-hour urine sample (basal), then 2 g of L-tryptophan in tablet form were given orally (day 1) and the subjects collected a second 24-hour specimen (post-tryptophan). A loading dose of 2 g of L-tryptophan also was administered on days 10, 20, 30, 40, 45, 51 and 54. A basal urine collection was made during the 24 hours preceding each of the loading tests and on these days no other metabolic testing was done.

All of the basal and post-tryptophan urine samples were analyzed for the following tryptophan metabolites: kynurenic acid and xanthurenic acid (13); indoxyl sulfate,[5] anthranilic acid glucuronide, o-aminohippuric acid, acetylkynurenine, kynurenine (14) and hydroxykynurenine (15). Paper chromatography and volatile amine determinations (14) were used to check the quantitative results. Statistical comparisons were made using Student's *t* test.

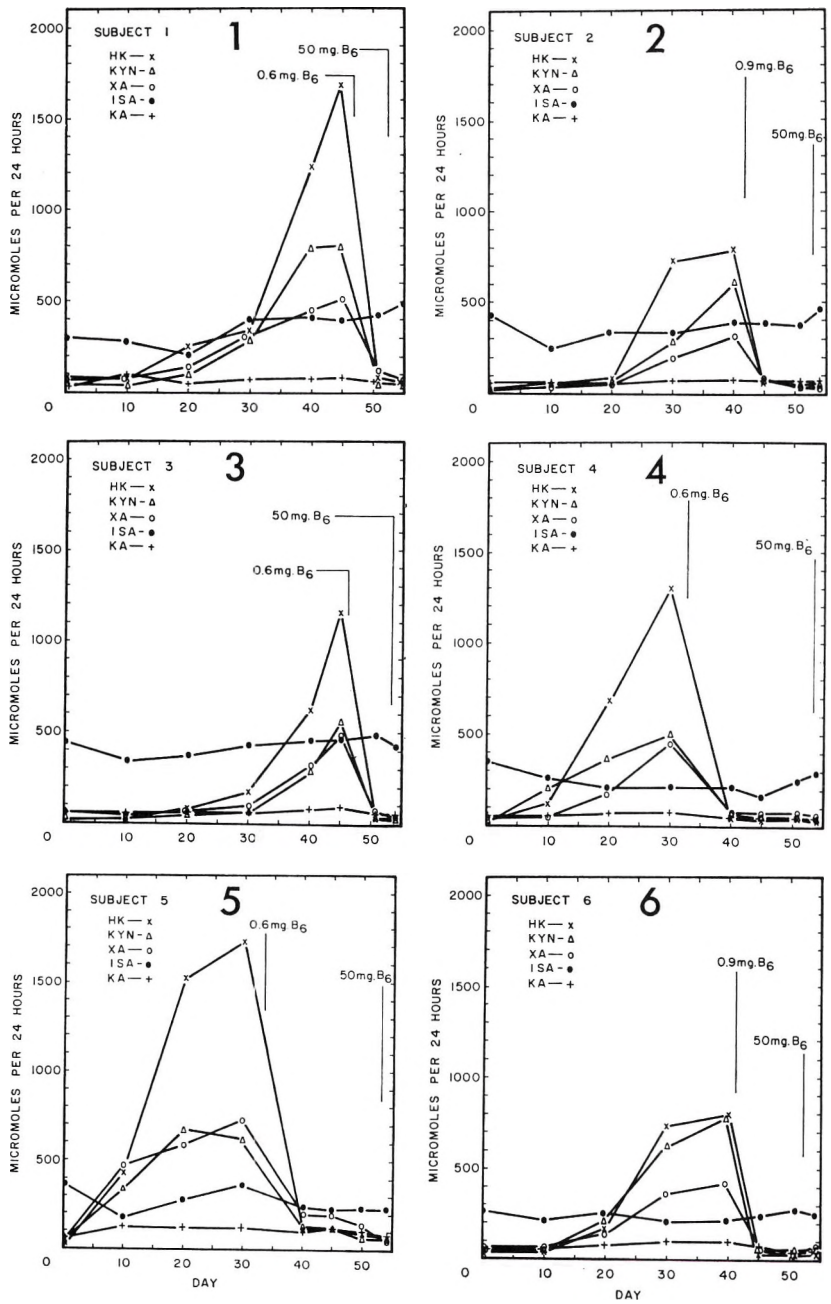
Supplemental pyridoxine at a level of 0.6 mg was administered, in addition to the 0.16 mg of dietary vitamin B_6, to subjects 4 and 5 beginning on day 33 and to subjects 1 and 3 beginning on day 46. Subjects 2 and 6 received 0.9 mg of supplemental pyridoxine commencing on day 42. These levels of pyridoxine were maintained throughout the remainder of the study until the last 3 days when all 6 subjects were given 50 mg of the vitamin daily.

RESULTS

The 6 subjects had normal basal and post-tryptophan levels of the urinary metabolites measured during the pre-depletion period; that is, the quantities of metabolites excreted by these 6 young men fell within the range of values previously found in this laboratory in a group of persons with no known disease (9). With the institution of the vitamin B_6-deficient diet, abnormal tryptophan metabolism following the 2-g loading dose became evident in all of the subjects but at varying rates and to different degrees (figs. 1–6). Two of the subjects (4 and 5) exhibited an abnormal response to tryptophan loading as early as day 10, whereas the remaining 4 subjects did not show this tendency until somewhat later. Although all of the subjects had some evidence of abnormal tryptophan metabolism by day 20, the deviations from normal varied greatly. For example, the post-tryptophan levels of hydroxykynurenine varied from 87 to 1521 μmoles at that time.

In addition to the dissimilarity in the time required for markedly abnormal tryptophan metabolism to appear, there was some variation in the relative magnitude of the quantities of metabolites which the subjects excreted during the initial stage of depletion. At the time abnormal metabolism first appeared (figs. 1–6), metabolites were excreted in largest amounts in the following order: kynurenine > hydroxykynurenine > xanthurenic acid (subjects 4 and 6); hydroxykynurenine > xanthurenic acid > kynurenine (subjects 1 and 3); hydroxykynurenine > kynurenine > xanthurenic acid (subject 2); xanthurenic acid > hydroxykynurenine > kynurenine (subject 5). Since these early changes were not large, and varied from subject to subject, little biochemical significance can be attached to them. Because of the wide variation in individual response to the deficient diet, the day on which pyridoxine supplementation was begun was based on the severity of the tryptophan metabolic abnormality. Therefore, those subjects whose metabolism became abnormal most rapidly, 4 and 5, were given the supplement first (day 33). As the metabolism of the remaining 4 men became more ab-

[5] Previously referred to as "fraction A" (13).



Figs. 1–6 Excretion values for hydroxykynurenine (HK), kynurenine (KYN), xanthurenic acid (XA), indoxyl sulfate (ISA) and kynurenic acid (KA) in subjects ingesting a pyridoxine-deficient diet. The values shown are micromoles excreted per day following an oral load of 2.0 g L-tryptophan and the vertical lines indicate when daily pyridoxine supplementation was started.

normal, they were given the supplement in pairs—subjects 2 and 6 on day 42 and subjects 1 and 3 on day 46.

The 2 days (basal and post-tryptophan) immediately preceding supplementation with pyridoxine have been termed the "height of depletion." This designates the point at which each subject evidenced his most abnormal response to tryptophan while consuming the vitamin B_6-deficient diet and therefore does not represent the same 2 days for all 6 subjects. At the height of depletion, the subjects were excreting significantly lower basal quantities of kynurenic acid and o-aminohippuric acid and higher quantities of kynurenine compared with their pre-depletion basal levels (table 1). After tryptophan loading, the subjects excreted significantly greater amounts of kynurenic acid, xanthurenic acid, acetylkynurenine, kynurenine and hydroxykynurenine and a significantly smaller amount of o-aminohippuric acid compared with the pre-depletion levels (table 2). As each pair of subjects was given supplemental pyridoxine, their tryptophan metabolism became more nearly normal as shown by a decrease in the excretion of the metabolites which had appeared in such large amounts in the urine during depletion. During the last 3 days of the study, when each subject was receiving 50 mg of pyridoxine/day, basal levels of kynurenic acid and xanthurenic acid were significantly lower and kynurenine significantly higher than during the pre-depletion period (table 1). Following tryptophan loading, xanthurenic acid was excreted in significantly smaller quantities than it had been after the initial base-line dose (table 2).

DISCUSSION

Other investigators (2–4, 8) have not reported variations in the rate at which subjects begin metabolizing tryptophan abnormally in response to a diet deficient in vitamin B_6. Differences which occurred among the subjects in the present study may have been partially due to their dietary intakes prior to participation in the experiment. Variations in the amounts of certain metabolites excreted during the first days of feeding the deficient diet may have been caused by depletion of specific enzyme systems at different rates. Since

TABLE 1

Average excretion of metabolites before tryptophan

Experimental time	Metabolite excreted [1]							
	KA	XA	ISA	AAG	o-AH	ACK	KYN	HK
	μmoles/24 hr		*μmoles/24 hr*		*μmoles/24 hr*		*μmoles/24 hr*	
Pre-depletion	18	11	315	3	27	8	7	17
Height of depletion	13 [2]	10	282	2	14 [2]	8	13 [3]	28
Day 50	15 [2]	11	345	4	24	8	13 [4]	26
Day 53	15 [2]	7 [2]	314	4	21	10	16 [2]	19

[1] The following abbreviations are used: KA, kynurenic acid; XA, xanthurenic acid; ISA, indoxyl sulfate; AAG, anthranilic acid glucuronide; o-AH, o-aminohippuric acid; ACK, acetylkynurenine; KYN, kynurenine; HK, hydroxykynurenine.
[2] Significantly different from the mean during the pre-depletion period at the 0.01 probability level.
[3] Significantly different from the mean during the pre-depletion period at the 0.05 probability level.
[4] Significantly different from the mean during the pre-depletion period at the 0.02 probability level.

TABLE 2

Average excretion of metabolites after tryptophan

Experimental time	Metabolite excreted [1]							
	KA	XA	ISA	AAG	o-AH	ACK	KYN	HK
	μmoles/24 hr		*μmoles/24 hr*		*μmoles/24 hr*		*μmoles/24 hr*	
Pre-depletion	54	41	361	6	48	9	22	21
Height of depletion	88 [2]	484 [2]	337	5	31 [2]	110 [2]	643 [2]	1294 [2]
Day 51	60	68	332	7	53	11	42 [2]	45 [3]
Day 54	53	28 [3]	343	6	42	13	30	31

[1] The following abbreviations are used: KA, kynurenic acid; XA, xanthurenic acid; ISA, indoxyl sulfate; AAG, anthranilic acid glucuronide; o-AH, o-aminohippuric acid; ACK, acetylkynurenine; KYN, kynurenine; HK, hydroxykynurenine.
[2] Significantly different from the mean during the pre-depletion period at the 0.01 probability level.
[3] Significantly different from the mean during the pre-depletion period at the 0.02 probability level.

these dissimilarities did not persist (that is, eventually all subjects excreted hdyroxykynurenine in greatest quantity, followed by kynurenine and xanthurenic acid) this appeared to be a transient phenomenon.

Previous studies in this laboratory have shown abnormal tryptophan metabolism in patients with such widely diverse conditions as scleroderma, bladder cancer, porphyria, pregnancy and drug-treated tuberculosis (9). An evaluation of data obtained from the tuberculosis patients receiving isoniazid or deoxypyridoxine revealed that significantly larger than normal amounts of hydroxykynurenine, kynurenine and acetylkynurenine were excreted following tryptophan administration (11).

Patients taking isoniazid excreted less than normal quantities of kynurenic acid; in the patients under treatment with deoxypyridoxine, kynurenic acid excretion was abnormally high. Kynurenine and xanthurenic acid excretions were almost equal in the patients ingesting deoxypyridoxine, whereas xanthurenic acid excretion was much less than kynurenine in the individuals given isoniazid. The yield of metabolites (post-tryptophan minus basal)

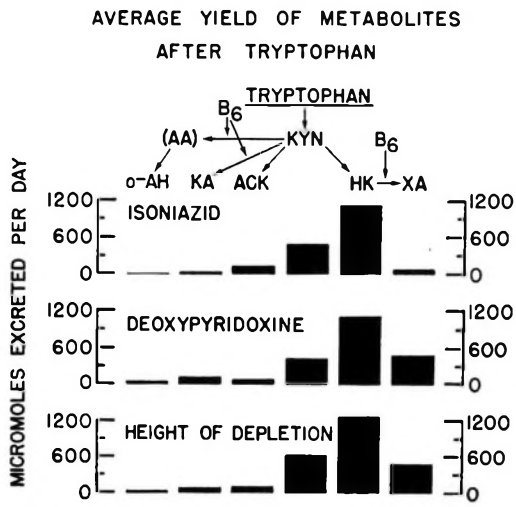


Fig. 7 Yield of metabolites (post-tryptophan value minus pre-tryptophan value) for subjects with vitamin B_6 deficiencies induced by isoniazid, deoxypyridoxine or dietary depletion. Abbreviations used are KYN, kynurenine; ACK, acetylkynurenine; KA, kynurenic acid; AA, anthranilic acid; o-AH, o-aminohippuric acid; HK, hydroxykynurenine; XA, xanthurenic acid. The symbol B_6 indicates known sites of action of pyridoxal phosphate.

in a patient who received deoxypyridoxine was remarkably similar to that of the subjects in the present study when they were at the height of depletion (fig. 7).

The tuberculosis patients receiving deoxypyridoxine excreted enough hydroxykynurenine to account for from 10 to 25% of the 2 g of L-tryptophan; in this study hydroxykynurenine accounted for 8 to 21% of the same quantity of tryptophan. Kynurenine, in the tuberculosis study, was excreted in amounts equivalent to 5 to 10% of the tryptophan load; in the nutritionally depleted subjects 5 to 8% of the tryptophan appeared as that metabolite. From these similarities it appears that vitamin B_6 deficiency induced by the administration of deoxypyridoxine and that produced by dietary means have a comparable effect on the vitamin B_6-dependent enzymes needed to metabolize tryptophan. In this nutritional investigation, kynureninase activity was apparently preferentially depressed over that of kynurenine transaminase allowing for the conversion of hydroxykynurenine to xanthurenic acid and of kynurenine to kynurenic acid. The apparent loss of kynureninase activity with little loss of kynurenine transaminase activity may be a demonstration of the variation in sensitivity of specific pyridoxine-requiring enzymes to pyridoxine deficiency which Snell (16) has pointed out. A decrease in kynureninase activity also would result in a decrease in the formation of anthranilic acid from kynurenine, and would be manifested by the lowered o-aminohippuric acid excretion which was observed during depletion. The levels of anthranilic acid glucuronide and indoxyl sulfate remained within normal limits throughout the entire study. The effect of vitamin B_6 depletion on the conversion of tryptophan to niacin via hydroxyanthranilic acid will be presented in a subsequent paper.

Korbitz et al. (17) reported elevated urinary levels of hydroxykynurenine, kynurenine, acetylkynurenine and xanthurenic acid in pyridoxine-deficient rats. Because these workers did not observe a corresponding increase in kynurenic acid, they supported the suggestion of Brown et al. (10) that the transamination of kynurenine and hydroxykynurenine may involve 2 distinct

enzymes. The observations of the current experiment do not refute this hypothesis since the low basal level of kynurenic acid and its relatively small increase post-tryptophan, compared with xanthurenic acid, at the height of depletion could be accounted for by kynurenine transaminase being more sensitive to vitamin B_6 deficiency than hydroxykynurenine transaminase. Korbitz et al. (17) also suggested the possibility that an alternate pathway which is not vitamin B_6-dependent may exist for the formation of xanthurenic acid.

The tryptophan metabolism of subjects whose pyridoxine deficiency has been induced by dietary means has been studied by other investigators and similar results were obtained. The study of Baker et al.,[6] in which urinary xanthurenic acid and hydroxykynurenine were measured, demonstrated high levels of these 2 metabolites as deficiency was produced. They reported that urinary xanthurenic acid was the best indicator of deficiency and that hydroxykynurenine appeared more sensitive than xanthurenic acid to variations in vitamin B_6 intake. In the present study, hydroxykynurenine was the most sensitive indicator of vitamin B_6 depletion of the tryptophan metabolites measured. Since no quantitative values were reported by Baker et al.,[7] a direct comparison of results cannot be made.

Several research groups have observed increased xanthurenic acid excretion following depletion of pyridoxine. Babcock et al. (2) noted that 7 male subjects eliminated an average of 42 mg of xanthurenic acid in response to a 5-g loading dose of L-tryptophan. In the study of Hodges et al. (8) one male excreted approximately 450 mg of xanthurenic acid and another, who was also receiving a vitamin B_6 antagonist, excreted approximately 350 mg xanthurenic acid after loading with 10 g of DL-tryptophan. Greenberg et al. (1) reported that 271 and 515 mg of the metabolite were produced by 2 subjects who had consumed a pyridoxine-deficient diet for 3 weeks. In Cheslock and McCully's experiment (3) the xanthurenic acid output of 5 females increased to more than 30 mg/day after they had eaten a low pyridoxine diet for 52 days. Knapp (4) reported average xanthurenic acid levels of about 160 mg after loading with 10 g of DL-tryptophan in subjects who had eaten a low pyridoxine diet for 3 weeks. The 6 males in the present study excreted an average of 74 mg of xanthurenic acid when a 2-g load of L-tryptophan was given after 25 days of depletion, and 99 mg at the height of depletion. Faber et al. (18), whose subjects were both pyridoxine- and pantothenic acid-deficient, reported an initial increase in xanthurenic acid excretion during the first 5 weeks of depletion, but at that point urinary xanthurenic acid decreased as the subjects showed marked nitrogen loss. As the men in the present study did not show decreasing levels of xanthurenic acid excretion (until supplemented with pyridoxine), it appears that the double vitamin deficiency or other experimental differences between the 2 investigations account for this discrepancy.

There is little evidence in the present data to support the concept of 3 stages of pyridoxine deficiency proposed by Kupke and Knapp (19). They proposed that in mild pyridoxine deficiency, excretion of kynurenine and hydroxykynurenine, but not of xanthurenic acid, would be elevated. Only in moderate vitamin B_6 deficiency would xanthurenic acid be excreted in elevated amounts along with kynurenine and hydroxykynurenine. In severe pyridoxine depletion, xanthurenic acid excretion would be decreased because of inhibition of the transamination reaction also. In general, in the present study, as well as in the study of Knapp (4), kynurenine, hydroxykynurenine and xanthurenic acid excretion became abnormal at the same time as the depletion developed. Furthermore, even at the height of deficiency, these 3 metabolites were excreted in elevated amounts with no definite evidence of decreasing xanthurenic acid excretion as predicted by Knapp. However, it is true that in isoniazid-treated patients the xanthurenic acid excretion is not elevated as much as expected (11), implying an inhibition of the transamination also. Certainly it is important to measure more metabolites than just xanthurenic acid if an adequate evaluation of tryptophan me-

[6] See footnote 4.
[7] See footnote 4.

tabolism and pyridoxine nutrition is desired.

Administration of the pyridoxine supplement to the subjects in this study caused a dramatic decrease in the urinary output of those metabolites which had been elevated during depletion (figs. 1–6). But it was not until 50 mg of vitamin B_6 were administered during the last 3 days of the study that the metabolite excretion of subject 5 returned to its pre-depletion status (fig. 5). This probably indicates a slightly higher requirement for vitamin B_6 by this individual than that being given (0.6 mg) prior to the 50-mg supplement. Subjects 1 and 3 received 0.6 mg of the vitamin beginning on day 46 (figs. 1 and 3). They were excreting increased quantities of xanthurenic acid until they were given 50 mg of pyridoxine. Their tryptophan metabolism might have returned to normal with a daily intake of 0.6 mg of vitamin B_6 if continued for a longer period of time.

The excretion of the majority of tryptophan metabolites had returned to predepletion levels by the end of the experiment. Two metabolites, kynurenic acid and xanthurenic acid, were lower than during the pre-depletion period. Babcock et al. (2) have also observed this phenomenon in the case of xanthurenic acid. They suggested that the vitamin B_6 intakes of their students were inadequate to meet the requirements for metabolizing 5 g of tryptophan plus the dietary protein. It appears unlikely that the same situation existed in this study with a 50-mg vitamin B_6 intake, a 2-g tryptophan load and 100 g of dietary protein. In view of the decreased basal levels of kynurenic acid and xanthurenic acid (table 1) and post-tryptophan xanthurenic acid (table 2) it appears more likely that selective repletion of the enzyme systems may play a role here.

Although abnormal excretion of tryptophan metabolites in pyridoxine deficiency is amply supported by experimental evidence, it is difficult to compare quantitative values from one study with another because of the various analytical methods used, the quantity of tryptophan administered and other differences in research design. The evaluation of work in this field would be facilitated if a standard technique was used for such studies (20). Collaborative studies such as this in which a number of parameters can be measured yield additional information concerning the essentiality of vitamin B_6 in tryptophan metabolism, although much remains to be learned relating a deficiency of the vitamin to specific enzyme changes. Knowledge in this area may be helpful in understanding the abnormal tryptophan metabolism observed in a variety of clinical conditions (9).

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Toxicity of Saturated Fat [1,2]

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ABSTRACT When diets containing high levels (20 to 40%) of either palmitate or stearate are fed to weanling mice, poor growth and high mortality result. Adult mice are similarly affected, although less severely. The addition to the diet of 4% of fats rich in either oleate or linoleate prevents the toxicity, whereas linolenic, palmitoleic and petroselinic acids are much less effective. The toxicity is increased slightly by the addition of cholesterol and very markedly when lactose constitutes the principal dietary carbohydrate. Depot fat levels of the dietary saturated fatty acid increase only slightly, particularly when compared with the changes observed when unsaturated fatty acids are fed. Digestibility studies preclude ascribing these effects to poor assimilation of the dietary fat.

The effect of dietary oleate and linoleate on the composition and distribution of fatty acids in the depot fat of mice has been previously investigated (1, 2). When similar studies were attempted with palmitate and stearate, poor growth and high mortality were encountered. Moreover, the depot fat levels of the saturated acids barely increased compared with expectations based on the studies with the unsaturated fatty acids. The results of these investigations provide the basis for the present report.

EXPERIMENTAL

The animals used in these experiments were either weanling or adult male mice obtained from the North Carolina State Laboratory of Hygiene. They were fed a diet containing 20% of casein, 5% of salt mixture W [3] and the remaining 75% composed of various mixtures of carbohydrate and fat. The diet was supplemented with all of the known vitamins [4] (3). The usual duration of an experiment was 3 weeks.

In the experiment in which the digestibility of the fat was determined, 12 adult mice were used. Digestibility was computed from the excretion of chromic oxide over a 5-day experimental period following a 5-day preliminary period. To facilitate the analysis of chromium, the radioisotope Cr^{51} was used and radioactivity was measured in a Nuclear-Chicago gamma scintillation spectrometer.

Triglycerides of the depot fat were isolated and their fatty acid composition determined by gas chromatography on a succinate-ethylene glycol polyester column (2).

RESULTS

Effect of saturated fat on weanling mice. Unlike results observed with unsaturated fat, as the dietary level of glycerylmonopalmitate and glycerylmonostearate was increased beyond 10%, there was a progressive decrease in growth and increase in mortality of the experimental animals (table 1). The toxic effect of glycerylmonopalmitate was more severe than glycerylmonostearate. Autopsy of animals that died revealed no gross abnormalities other than that of emaciation. In most instances the adipose tissue was completely devoid of fat, although the stomach and intestines contained considerable quantities of food and the animals continued to eat until the day of death.

Bosshardt et al. (4) reported toxicity of diets containing 20% of palmitic acid, 1% of cholesterol and 0.05 to 0.15% of oleic acid when fed to weanling mice. Omission of either the cholesterol or oleic acid alleviated the toxic effects encountered in the mixture with male mice, whereas 20% of

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[3] Nutritional Biochemicals Corporation, Cleveland.

[4] Kindly furnished by Merck and Company, Inc., Rahway, New Jersey.

TABLE 1

Effect of glycerylmonopalmitate and glycerylmonostearate on growth and mortality of weanling mice [1]

Dietary level	Glycerylmonopalmitate		Glycerylmonostearate	
	Wt gain	Mortality	Wt gain	Mortality
%	g	%	g	%
0	2.0	0	1.1	0
5	3.2	0	1.2	0
10	2.1	0	2.1	0
20	− 6.2	40	0.9	0
30	− 5.6	40	− 0.3	0
40	—	100	− 1.1	0
50	—	—	− 8.6	17

[1] Duration of experiments 3 weeks. Five mice were used for each dietary level of glycerylmonopalmitate and 6 mice for each dietary level of glycerylmonostearate.

palmitic acid alone adversely affected the growth and mortality of weanling female mice. Postmortem examination revealed small spleens as the only gross abnormality. The results of the experiment of table 1 differ from those of Bosshardt et al. (4) chiefly in that the toxic effects of saturated fats on male mice were obtained without the addition of cholesterol, and thus resemble their observations with female mice. The oleum percomorph used as a source of the fat-soluble vitamins could have contributed the trace of oleic acid these investigators (4) found to be required for toxicity.

Effects of unsaturated fats on the toxicity of saturated fat. Although the diets used in this study contained no added source of essential fatty acids (other than the oleum percomorph), no sign of a deficiency of this essential nutrient had appeared in 3 weeks in previous investigations with fat-free diets of similar composition (1–3). However, since the addition of saturated fat to the diet has been shown to promote an essential fatty acid deficiency (5), it appeared possible that the lack of growth and high mortality encountered with the high levels of glycerylmonopalmitate and glycerylmonostearate might represent an acute form of this deficiency disease. On the other hand, Bosshardt et al. (4) had noted that increasing the dietary level of oleic acid reversed the toxicity of their saturated fat diet.

With weanling mice both oleic acid and glycerylmonooleate were as effective as either safflower oil or the fatty acids from safflower oil (table 2) in preventing the toxicity of the saturated fat. Therefore, the toxicity of the saturated fat is not merely an uncomplicated essential fatty acid deficiency. Since free fatty acids, monoglycerides and triglycerides are all effective, the glyceride form of the unsaturated fatty acid is of no consequence in the prevention of the toxicity of glycerylmonopalmitate. The failure of either polyoxyethylene sorbitan monopalmitate (Tween 40), polyoxyethylene sorbitan monooleate (Tween 80) or paraffin oil to effect a reversal of the toxicity of the saturated fat indicates that the action of the unsaturated fats is more than a mere alteration of the physical consistency of the dietary fat.

Although the effects are less pronounced, the diet containing 40% of glycerylmonopalmitate is also toxic to adult mice (table 2). As with weanling mice, the addition of a fat rich in unsaturated fatty acids is effective in preventing the toxicity of the saturated fat in the adult animal. In contrast, supplementation of the diet with coconut oil was less effective, whereas α-tocopheryl acetate gave no protection whatsoever.

In an experiment using free palmitic acid rather than the monoglyceride, the study of the reversal of the toxicity of saturated fat by unsaturated fat was extended to other unsaturated fatty acids. Although the free palmitic acid when fed at a level of 40% proved more toxic than 40% of glycerylmonopalmitate (equivalent to 31% of palmitic acid), the inclusion of 4% of safflower oil in the diet was effective in reversing the toxic effects of the higher intake of saturated fatty acid (table 3).

TABLE 2

Reversal of toxicity of glycerylmonopalmitate by unsaturated fat [1]

Diet supplement	No. mice	Wt gain	Mortality
		g	%
	Weanling mice		
None	23	− 0.1	74
4% Safflower oil	17	3.2	6
4% Safflower oil fatty acids	6	4.6	17
4% Oleic acid	6	4.6	0
4% Glycerylmonooleate	11	2.0	18
4% Tween 40 [2]	5	—	100
4% Tween 80 [3]	6	− 1.4	67
2% Paraffin oil	5	− 1.2	60
	Adult mice		
None	6	− 1.9	33
4% Safflower oil	6	0.1	0
4% Corn oil	6	0.1	0
4% Olive oil	6	1.0	17
4% Lard	6	1.0	0
4% Coconut oil	6	− 0.7	17
0.1% α-Tocopheryl acetate	6	− 3.2	50

[1] Duration of experiments 3 weeks. All diets contained 40% of glycerylmonopalmitate.
[2] Polyoxyethylene sorbitan monopalmitate, Atlas Powder Company, Wilmington, Delaware.
[3] Polyoxyethylene sorbitan monooleate.

TABLE 3

Effect of unsaturated fatty acids on the reversal of toxicity of palmitic acid [1]

Unsaturated fatty acid	No. mice	Mortality
		%
None	11	100
Linoleic	10	20
Linolenic	12	83
Palmitoleic	10	60
Petroselinic	10	70

[1] Duration of experiments 2 weeks. Diets contained 40% of palmitic acid and 4% of the unsaturated fatty acid. Linoleic acid was fed as safflower oil. Data given are the average of 2 experiments.

In contrast, neither linolenic acid, palmitoleic acid nor petroselinic acid, when added to the diet, was capable of preventing the toxicity of the saturated fat.

The results of a study to ascertain the dietary level of safflower oil required for prevention of the toxicity of glycerylmonopalmitate are shown in figure 1. As the level of safflower oil in the diet increased, there was a decrease in the mortality of the animals and an increase in the growth of those animals that survived. Since 4% of safflower oil was about as effective as 8%, it is apparent that a small quantity of unsaturated fat will alleviate the effects of a tenfold amount of saturated fat.

Effect of cholesterol on the saturated fat toxicity. In the experiments of Bosshardt et al. (4) with male mice ingesting a diet containing 20% of palmitic acid, the adverse effects caused by the saturated fat were not observed unless cholesterol was included in the diet. Although dietary cholesterol was not required to produce the toxic effects of the saturated fat when the diet contained a higher level of palmitic acid (table 2), it appeared likely that the addition of cholesterol might accentuate the toxic effects obtained with the higher level of saturated fat. The results of a study designed to test this hypothesis (table 4) showed that cholesterol did enhance the toxic effects of palmitate. This is noticed most strikingly in the diets containing 2% of safflower oil where cholesterol addition doubled the mortality and greatly reduced the growth of the animals that survived. When, however, the level of safflower oil was increased to 4%, only a slight difference in growth rate was observed, and even this disappeared when 8% of unsaturated fat was included in the diet.

Effect of carbohydrate on toxicity of saturated fat. When the sucrose component of the diet was replaced by glucose, no effect on the toxicity of the glycerylmonopalmitate was observed (table 5). When, however, lactose was used as the dietary carbohydrate, the toxicity of the

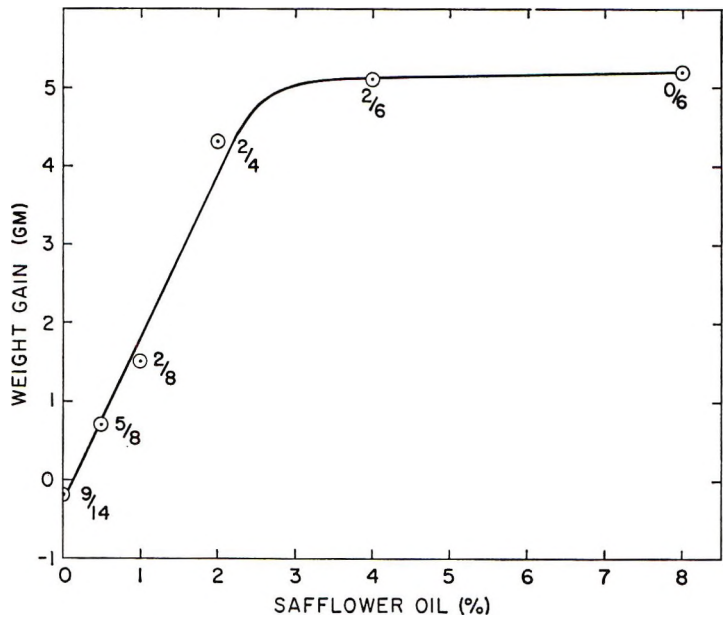

Fig. 1 Effect of safflower oil supplementation on toxicity of glycerylmonopalmitate. Basal diet contained 40% glycerylmonopalmitate. Data are 3-week weight gain of survivors of 2 experiments with weanling male mice. Fractions show mortality and total number of mice for each level.

TABLE 4

Effect of cholesterol on toxicity of glycerylmonopalmitate [1]

Safflower oil	Cholesterol			
	None		1%	
	Wt gain	Mortality	Wt gain	Mortality
	g	%	*g*	%
None	—	100	−3.5	83
2%	4.2	33	1.2	67
4%	5.1	33	3.3	33
8%	5.2	0	5.7	0

[1] Duration of experiment 3 weeks with 6 mice/treatment. All diets contained 40% of glyceryl-monopalmitate.

TABLE 5

Effect of carbohydrate on toxicity of glycerylmonopalmitate [1]

Carbohydrate	1 Week		3 Weeks	
	Wt gain	Mortality	Wt gain	Mortality
	g	%	*g*	%
Sucrose	−1.1	25	−0.2	37
Glucose	−0.1	37	2.0	50
Lactose	—	100	—	—

[1] Diets contained 40% of glycerylmonopalmitate and 35% of the carbohydrate indicated. Each diet was fed to 6 mice.

TABLE 6

Toxicity of glycerylmonopalmitate in the presence of lactose

Diet addition	GMP [1] level	No. mice	Wt gain	Mortality
	%		g	%
None	0	12	1.7	42
None	10	6	5.2	17
None	20	18	4.2	83
None	40	12	—	100
4% Safflower oil	20	12	2.6	25
8% Safflower oil	20	12	4.2	25
8% Safflower oil	40	6	2.6	67
40% Lard	0	6	7.7	0

[1] Glycerylmonopalmitate.

diet became much more acute, diarrhea developed and none of the animals survived the first week of the experiment. Autopsy of these animals disclosed marked intestinal edema, in addition to the emaciation previously observed with the high level of saturated fat.

Because the adipose tissue of animals that ingest lactose becomes depleted of fa (6), as is the case for mice fed diets rich in saturated fat, the interrelationship between these 2 dietary ingredients was investigated further. When a diet containing 35% of lactose and no fat was fed, high mortality and poor growth were observed (table 6). These results are consistent with the observations of other investigators (7). The addition of 10% of glycerylmonopalmitate resulted in marked improvement in both growth and survival. When, however, the level of saturated fat was increased to 20%, a mortality double that encountered with the diet containing no added fat was observed, although the growth of the animals that survived was not different from those fed the diet containing 10% of fat. Finally, when the saturated fat level was increased to 40%, complete mortality was observed before the end of one week. The addition of safflower oil reduced the toxicity of these diets (table 6), but the unsaturated fat was not as effective as in diets when lactose was absent (fig. 1). In contrast with the diets containing high levels of saturated fat, good growth and survival were obtained when the mice were given a diet containing 40% of lard.

Digestibility of saturated fat. It has long been recognized that saturated fats are less digestible than unsaturated fats, and that increasing the fluidity of a saturated fat by the addition of an unsaturated fat enhances its digestibility (8). In view of the syndrome of the saturated fat toxicity, it appeared possible that caloric insufficiency resulting from the low absorption of the saturated fat might account for the results observed. The rather high digestibility of the saturated fat diets (table 7) leads to a rejection of this hypothesis.

TABLE 7

Food consumption and digestibility of diets containing glycerylmonopalmitate (GMP)

Diet	Consumption	Fat digestibility
	g/mouse/day	%
40% GMP	2.8	81.6
40% GMP + 4% safflower oil	3.1	92.2

Although the addition of 4% of safflower oil increased the absorption of fat, the additional calories absorbed could hardly account for the marked effect of the unsaturated fat on survival and growth. Because of the method of extraction used, it is possible that not all of the fecal lipids were removed and that the fat digestibility figures of table 7 are higher than the true values. If one assumes, however, that the feces consisted entirely of fat, the fat digestibility would be 53 and 66% for the saturated fat diet alone and supplemented with safflower oil, respectively. Therefore, it is clear that mice are capable of absorbing an appreciable amount of the palmitate as well as a major proportion of the energy contained in the diet.

Deposition of saturated fatty acids. It has been long established that when an

animal ingests an unsaturated fat, the unsaturated fatty acids of the depot fat increase proportionately. Indeed, previous studies had shown that dietary oleate and linoleate are deposited in the depot fat of mice (1). It had been the initial objective of this investigation to extend the study of the effect of dietary fat on depot fat to palmitic and stearic acids. Therefore the fatty acid composition of the depot fat of the animals that survived the initial experiments (table 1) was determined by gas chromatography. In contrast with expectations, very little increase in the depot fat level of either palmitate or stearate resulted from the inclusion of the monoglycerides of these fatty acids in the diet (fig. 2).

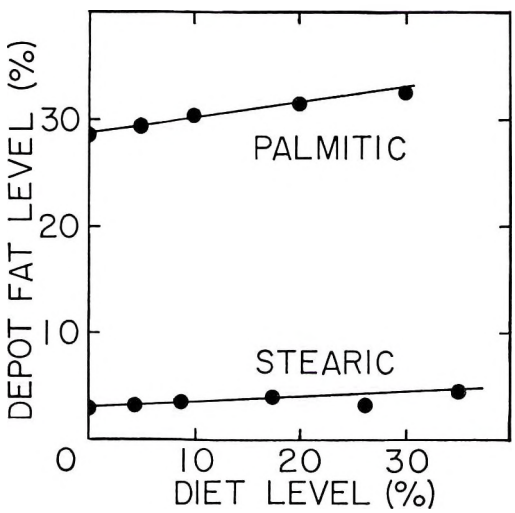

Fig. 2 Effect of dietary palmitic and stearic acids on their levels in depot fat. Fatty acids were fed as the monoglycerides. Data are derived from the survivors of the experiment described in table 1.

The magnitude of the differences between the effect of unsaturated fatty acids and saturated fatty acids on the composition of depot fat can best be illustrated by comparing the slopes of their response curves. The depot fat level of palmitic acid increased 0.06% for each percentage increase of palmitate in the diet, whereas for stearate the slope was only 0.03% (fig. 2). In contrast, for each percentage increase of oleate or linoleate in the diet the percentage in the depot fat increased more than 1 and 2%, respectively (1). These observations relate to the net change in the level of saturated fatty acids in the depot fat and do not mean that the dietary palmitate and stearate per se are deposited only slightly. The lack of increase in the level of saturated fatty acids could arise equally if the synthesis, or deposition, or both, of these acids from endogenous sources was inhibited by the influx of preformed saturated fatty acids from the diet. An indication that the deposition of endogenous saturated fatty acids may be inhibited is derived from the fact that the deposition of dietary pentadecanoic acid and dietary heptadecanoic acid was observed to be greater than the increase in palmitate deposition, although not as great as the increase obtained when the unsaturated fatty acids were fed.[5]

DISCUSSION

When high levels of saturated fat were fed to mice, poor growth and high mortality resulted. The only major outward manifestation of this toxicity was emaciation as evidenced by a depletion of the adipose tissue of the mice. This toxic condition can be prevented by the addition to the diet of a relatively small amount of oleate or linoleate. These observations are similar to those of Bosshardt et al. (4) who reported toxicity of diets containing 20% palmitic acid and 1% cholesterol. The latter investigators did not obtain the toxic effects in the absence of cholesterol in male mice but did in female mice. From the data reported herein it is apparent that increasing the dietary level of palmitic acid eliminates the need for cholesterol, although the addition of cholesterol increases the toxicity of these diets.

Adverse effects of feeding diets rich in saturated fatty acids are well established. The addition of saturated fat to diets deficient in linoleic acid decreases the time required for the onset of the essential fatty acid deficiency symptoms and increases their severity (5). Since, however, oleic acid is as effective as linoleic acid in reversing the toxicity of the saturated fat (table 2), the toxicity is not merely an uncomplicated essential fatty acid deficiency. On the other hand, there is some specificity associated with the reversal, in that oleic

[5] Tove, S. B., unpublished observations.

acid and linoleic acid are both equally effective, whereas linolenic acid, petroselinic acid and palmitoleic acid are much less effective. Herting et al. (9) noted that a foreign-body type of reaction in fat cells, lipogranuloma, developed when rats were given diets rich in saturated fat. They also observed that the lesions could be prevented by the addition of unsaturated fat to the diet. However, the level required (50% of the total) was much greater than that observed in our experiments (table 2). Although gross examination of the adipose tissue of the mice revealed no sign of the lipogranuloma, it is possible that the saturated fat toxicity reported herein represents an acute form, and the lipogranuloma described by Herting et al. (9), a chronic form of the same metabolic defect.

The mechanism by which the saturated fat produces the toxicity is unknown. The fat depletion of the adipose tissue indicates that the animals were not capable of meeting their energy needs. Yet the mice consume a diet until the day they die, and from the digestibility coefficient it is apparent they are receiving adequate calories from their diet. The high level of saturated fat in the diet may promote uncoupling of oxidative phosphorylation. Oxidative phosphorylation of liver mitochondria is uncoupled in essential fatty acid deficiency (10).

It is generally considered that when any fatty acid is consumed by an animal, that fatty acid increases in the depot fat. This conclusion is based largely on work with unsaturated fatty acids and with short-chain fatty acids such as those in coconut oil. For these fatty acids the statement has been amply proved (11). In contrast, from our results, the addition of palmitate or stearate to the diet does not produce a major increase in the levels of these acids in the adipose tissue. Although saturated fatty acids are not as digestible as unsaturated fatty acids, the digestion coefficients (table 7) indicate that low assimilation cannot entirely account for the failure of the level of palmitic acid in the depot fat to rise with increased dietary level. The digestibility of glycerylmonostearate, although lower than that of the palmitate (12), also could not entirely account for the low deposition of dietary stearic acid. In this respect, it is of interest to note that it is possible to increase the stearate level of the depot fat of mice more by feeding linoleic acid (1) than by feeding stearic acid.

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Effect of Dietary Fatty Acids on Assimilation of Fatty Acids by Adipose Tissue in vitro [1,2]

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ABSTRACT To study the effect of fatty acid composition of adipose tissue on assimilation of fatty acids in vitro, epididymal fat pads from mice fed various fats were incubated with C^{14}-labeled myristic, palmitic, stearic, oleic and linoleic acid, each in combination with 9,10-H^3-palmitic acid. Although feeding the various fats produced wide differences in fatty acid composition of the adipose tissue, there was no effect of dietary treatment on the incorporation of the labeled fatty acids into either the triglycerides or the β-position of the triglycerides. In each case the H^3:C^{14} ratio was lower for the fatty acids at the β-position than the triglycerides as a whole, thus providing evidence for the desaturation of palmitic acid prior to esterification at the β-position.

It has been shown (1, 2) that when a particular fatty acid in depot fat is increased by dietary means, the rest of the fatty acids do not maintain the same percentage distribution relative to each other. For example, when the percentage of linoleic acid in depot fat is increased, there is a decrease in the relative level of palmitoleic acid and an increase in the relative level of stearic acid (2). Similarly an increase in oleic acid level is associated with a decrease in the relative level of palmitic acid (2). The mechanism accounting for these specific patterns of substitution is unknown. One prominent possibility is that the esterification of a partial glyceride is specifically influenced by the fatty acid composition of that glyceride. This hypothesis was examined by incubating C^{14}-labeled fatty acids with epididymal fat pads obtained from mice fed various fatty acids. The results of this investigation provide the basis for the present report.

EXPERIMENTAL

Adult male mice obtained from the North Carolina State Laboratory of Hygiene were fed a sucrose-casein purified diet (3) for 4 weeks. The following fats were added at the expense of sucrose: 10% of glycerylmonomyristate, 30% of palmitic acid, 30% of glycerylmonostearate, 10% of triolein or 25% of safflower oil. The fatty acid composition of these fats was determined by gas chromatography (2) and is listed in table 1.

At the termination of the feeding period, the mice were decapitated and the epidymal fat pads rapidly excised and placed in cold Krebs-Ringer phosphate buffer, pH 7.4, with calcium chloride omitted. A single fat pad was selected at random from a pool of tissue from mice receiving the same diet; the pad was blotted dry, weighed and assigned to a given incubation flask. Incubations in duplicate were carried out in 50-ml Erlenmeyer flasks containing 2 ml of Krebs-Ringer phosphate buffer, pH 7.4 (calcium-free), on a shaking water bath at 37° for 2 hours. The substrate consisted of 2 μmoles of a fatty acid labeled with C^{14} in the carboxyl group and 2 μmoles of 9,10-H^3-palmitic acid. The fatty acids were added as a serum albumin complex prepared as described by Neptune et al. (5). A 3% solution of bovine serum albumin (fraction V) was prepared in Krebs-Ringer phosphate and dialyzed overnight against the same buffer prior to preparation of the fatty acid complex.

Analytical procedures. At the termination of the incubation the adipose tissue was removed from the flask, washed in a mixture of ethanol-ether (3:1), blotted dry

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TABLE 1

Fatty acid composition of fat supplement to purified diet fed mice

Chief dietary fatty acid [1]	Component fatty acid [1]						
	14:0	14:1	16:0	16:1	18:0	18:1	18:2
	%	%	%	%	%	%	%
14:0 [2]	97.7	—	2.2	—	—	—	—
16:0 [3]	0.4	1.0	91.0	0.7	7.0	—	—
18:0 [4]	1.7	2.6	25.6	1.7	67.5	—	—
18:1 [5]	3.6	1.4	5.0	6.7	1.1	74.7	6.5
18:2 [6]	—	—	7.7	—	3.9	13.6	74.8

[1] The first number indicates the number of carbon atoms, the second the number of double bonds.
[2] Glycerylmonomyristate prepared from myristic acid.
[3] Palmitic acid supplied by Armour Industrial Chemical Company, Chicago.
[4] Glycerylmonostearate, MYVEROL type 18-07, kindly furnished by Distillation Products Industries, Rochester, New York.
[5] Glyceryltrioleate, Emery 2230, kindly furnished by Emery Industries, Cincinnati, Ohio.
[6] Safflower oil kindly furnished by the Pacific Vegetable Oil Corporation, San Francisco.

and homogenized in 30 volumes of the solvent mixture. Following filtration, additional ether was added and the solution washed with water and dried over a mixture of anhydrous sodium sulfate and anhydrous sodium carbonate (4:1). The free fatty acids were removed by passage through a column of IRA 400 resin in the hydroxyl form (6). The effluent solution, showing a single triglyceride spot when examined by thin-layer chromatography (7), was divided into 4 portions. One portion was used for the determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation medium consisted of toluene containing 0.5% of diphenyloxazole and 0.03% of 1,4-bis-2-(5-phenyloxazolyl)-benzene. The amount of C^{14} or H^3 present was computed by the isotope ratio method of Okita et al. (8). A second portion was used for the determination of ester value (9). Aliquots of the triglycerides from animals receiving the same diet were pooled and the fatty acid composition determined by gas chromatography (2) following formation of the methyl esters of the fatty acids by transesterification (4).

The fourth portion of the triglyceride was subjected to hydrolysis by pancreatic lipase for 15 minutes (4) and the resulting monoglycerides isolated by Florisil chromatography (10) after removal of the free fatty acids by IRA 400 resin (6). The monoglycerides, representing the fatty acids of the β-position, were divided into aliquots for the determination of radioactivity, ester value and fatty acid composition. As with the triglycerides, gas chromatography was carried out on a pooled sample from animals on the same regimen. The purity of the monoglyceride fraction was examined by thin-layer chromatography (7) and a single spot migrating as monoglyceride was observed.

RESULTS

The feeding of the various fats did not greatly affect the body weight of the mice during the 4-week feeding period (table 2). In contrast, however, the average weight of the epididymal fat pad was materially influenced by the fat fed (table 2). The feeding of the saturated fatty acids resulted in a lower weight of the epididymal fat pad than did the feeding of either triolein or safflower oil. The effect of ingestion of a saturated fat was most strikingly evidenced in the case of palmitic acid where an almost complete absence of depot fat was observed. This marked effect of palmitic acid, as compared with that of myristic acid or stearic acid, may be related in part to the fact that a higher level of palmitic acid was fed and that the palmitate was given as the free acid, whereas the other saturated acids were fed as the monoglycerides. The reduced weight of adipose tissue that resulted from the feeding of saturated fat is a manifestation of the syndrome of saturated fat toxicity in mice (3).

The fatty acid composition of the pooled glycerides from animals fed the same diet is shown in table 3. In each case the percentage of the principal dietary acid in the depot fat increased over that observed in the depot fat of mice fed low fat diets (4),

TABLE 2

Effect of dietary fat on body weight and epididymal fat pad weight in mature mice [1]

Chief dietary acid [2]	Diet fat level	Body wt change	Epididymal fat pad wt
	%	*g*	*mg*
14:0	10	2.3	298 ± 17 [3]
16:0	30	−1.0	0 [4]
18:0	30	−0.1	236 ± 21
18:1	10	1.2	348 ± 25
18:2	25	1.0	354 ± 16

[1] Body weight changes are the average of 10 mice and epididymal fat pad weight is the average of 10 pads.
[2] The first number indicates the number of carbon atoms, the second the number of double bonds.
[3] SD of mean.
[4] Insufficient sample to easily excise was observed with all mice.

TABLE 3

Effect of dietary fat on the fatty acid composition and distribution of epididymal fat pads [1]

Component fatty acid [2]	Chief dietary fatty acid							
	14:0		18:0		18:1		18:2	
	TG [3]	β [4]	TG	β-	TG	β-	TG	β-
	%	%	%	%	%	%	%	%
14:0	5.6	16.1	2.1	13.1	0.9	29.4	0.9	41.7
16:0	18.8	16.1	22.5	13.5	15.8	11.9	14.7	41.7
16:1	11.5	38.4	12.9	33.3	10.0	26.3	4.5	35.7
18:0	9.8	1.4	4.2	6.5	2.3	5.4	3.9	45.4
18:1	41.0	45.4	49.4	41.7	59.5	38.5	28.2	41.7
18:2	5.7	83.3	7.3	55.5	9.3	62.5	46.4	21.7

[1] Fatty acid composition based on a pooled sample of an equal portion of triglyceride from each fat pad of mice receiving the same diet.
[2] The first number indicates the number of carbon atoms, the second the number of double bonds.
[3] Triglyceride fatty acid composition.
[4] Grams of the component fatty acid in the β-position per 100 g of that fatty acid in the total triglycerides.

although for stearate only a slight change was observed. The high level of stearate in the glycerides of the adipose tissue of mice fed glycerylmonomyristate is not usually observed with this diet [4] and cannot be accounted for in this experiment.

The feeding of glycerylmonomyristate, glycerylmonostearate or triolein did not alter appreciably the glyceride distribution of the component fatty acids (table 3) from that normally observed for depot fat obtained from mice that ingest low fat diets (4). The saturated fatty acids were predominantly esterified at the α-positions, the C^{18} unsaturated fatty acids were predominantly esterified at the β-position, and palmitoleic acid tended to be uniformly distributed. In contrast, the feeding of safflower oil resulted in the esterification of the major portion of linoleic acid at the α-positions (table 3). Thus the large influx of dietary linoleate into the triglycerides of the adipose tissue displaced the fatty acids normally esterified at the α-positions with the result that disproportionate esterification of the saturated fatty acids was reversed from a predominantly α-distribution to a predominantly β-distribution.

The effect of the nature of the dietary fat on the assimilation of various fatty acids by epididymal fat tissue in vitro is shown in table 4. To minimize animal variation, each fatty acid, labeled with C^{14} in the carboxyl group, was incubated with H^3-labeled palmitic acid. The results are expressed as the ratio of the specific activity of H^3-palmitic acid to that of a C^{14} acid. Although the results were variable, statistical analysis (11) revealed that dietary treatment did not significantly ($P < .05$) influence the uptake of any fatty acid. Moreover, neither prior dietary treatment nor structure of the companion C^{14}-labeled

[4] Tove, S. B., unpublished observations.

TABLE 4

Effect of diet on the ratio of H^3-palmitic acid to C^{14}-labeled fatty acids assimilated by epididymal fat pads in vitro

Medium C^{14}-acid	Chief dietary acid [1]				Avg all diets	
	14:0	18:0	18:1	18:2	Triglycerides	β-position
14:0 [2]	4.1	4.0	3.6	5.0	4.18 ± 0.20 [3]	3.41 ± 0.25
16:0	1.6	1.5	1.8	1.4	1.56 ± 0.02	0.86 ± 0.32
18:0	1.3	1.0	1.5	1.2	1.23 ± 0.09	1.02 ± 0.09
18:1	2.8	2.2	3.3	3.0	2.83 ± 0.28	1.45 ± 0.05
18:2	1.8	2.2	1.8	2.0	1.93 ± 0.08	0.77 ± 0.01

[1] Each value is the average of the ratio between the triglycerides of 2 incubations.
[2] The first number indicates the number of carbon atoms, the second the number of double bonds.
[3] SD of mean.

fatty acid had any effect on the assimilation of 9,10-H^3-palmitic acid.

Dietary treatment did not affect the incorporation of any C^{14}-labeled fatty acid into the β-position of the triglycerides (table 4). In each case, however, the ratio of H^3 to C^{14} was lower in the β-position than that of the triglycerides as a whole. This is particularly apparent for the unsaturated fatty acids and, unexpectedly, for C^{14}-labeled palmitic acid.

DISCUSSION

The primary purpose of this investigation was the examination of the hypothesis that the fatty acid composition of the glycerides of adipose tissue would exert an influence on the assimilation of fatty acids by the adipose tissue. The results revealed that this was not the case. This was most strikingly evident with linoleic acid where no difference in the assimilation of exogenous fatty acids was observed despite an eightfold range in linoleate content of the adipose tissue. A similar lack of fatty acid specificity was observed by Goldman and Vagelos (12) with a particulate enzyme preparation from chicken adipose tissue that catalyzed the formation of triglycerides from fatty acyl-CoA esters and diglycerides. Thus the data of this investigation, as well as the observations of Goldman and Vagelos (12), provide little evidence that the presence of a given fatty acid in a partial glyceride exerts an influence over the particular fatty acid with which the glyceride is esterified further.

The observation that the 9,10-H^3-labeled palmitic acid was esterified at the β-position to a lesser extent than the C^{14}-labeled fatty acids, especially palmitic-1-C^{14} acid, could be ascribed to desaturation of the palmitic acid at the 9,10-position prior to incorporation at the β-position. Shapiro and Wertheimer (13) have demonstrated the presence of a fatty acid desaturase system in adipose tissue capable of dehydrogenating palmitic acid to form palmitoleic acid. The apparent preference of the desaturated fatty acid for the β-position is confirmation of the observation that fatty acids occupying the β-position come from different pools than those fatty acids esterified at the α-positions (14).

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Relationships between Biotin and Vitamin B_{12}

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ABSTRACT Interrelationships between biotin and vitamin B_{12} were studied. Data indicate that vitamin B_{12} administration to rats receiving a biotin-free diet exerts a favorable effect on the growth rate and on the delay of the deficiency symptoms in these experimental conditions. Vitamin B_{12} appears also to have an effect on transcarbamylase activities analogous to biotin, possibly due to an increased intestinal synthesis of biotin and tissue storage. In addition, biotin administration influences methylation examined in the rat in vivo (urinary elimination of N-methylnicotinamide) and in vitro (betaine-homocysteine transmethylase) as does vitamin B_{12} although to a lesser extent. The significance of the interrelationship between vitamins is discussed.

The relationships between biotin and other vitamins have been the subject of numerous studies (1). Riboflavin has been observed to exert a favorable action on the synthesis of biotin in the intestinal tract of the rat. Pyridoxine according to some authors reduces the severity of the deficiency symptoms, whereas according to others it has no protective effect. The addition of thiamine to the culture medium enhances the synthesis of biotin by *Phycomyces blakesleeanus*, and a marked decrease in thiamine concentration is observed in the liver of the biotin-deficient rat. Finally biotin has been shown to exert a protective effect against pantothenic acid deficiency. However, these data are incomplete, and have not always been confirmed or definitely elucidated.

The interrelationships between biotin and ascorbic acid are more evident and have been demonstrated. Ascorbic acid administered to biotin-deficient rats cures almost all the specific symptoms of this deficiency. The mechanism of this "sparing" action appears to be related to the redox properties of ascorbic acid, because other redox agents, devoid of any vitamin activity, show a similar, though less effective, activity.

There are no data in the literature concerning the relationships between biotin and vitamin B_{12} except in propionic acid metabolism (2). Therefore the present experiments were carried out to investigate the interrelationships between vitamin B_{12} and biotin. The effects of vitamin B_{12} administration to rats fed a biotin-deficient diet were studied with respect to growth rate and development of deficiency symptoms, biotin and vitamin B_{12} levels in the liver, vitamin B_{12}-dependent enzymatic activities and finally enzymatic activities on which biotin appears to exert some effect.

EXPERIMENTAL

In experiments 1 and 2 weanling male rats of the Wistar strain were divided into 3 groups and housed in cages with wire bottoms. The rats of groups 1 and 3 were fed a biotin-free diet having the following composition: vitamin-free casein, 20; sucrose, 59; salt mixture no. IV (3), 4; groundnut oil, 5; raw dried egg white, 11; vitamin mixture,[1] 1. Group 2 was fed the same diet but the raw dried egg white was replaced by an egg white autoclaved at 120° for 15 minutes and dried. Biotin (100 μg/kg) and vitamin B_{12} (100 μg/kg) were added to the diets of groups 2 and 3, respectively. In experiment 3 a fourth group of rats maintained with the biotin free-diet and fed a supplement of both biotin and vitamin B_{12} served as normal controls. After 60 days, 8 animals per group of experiments 1 and 2 were used for the determination of the methylation capacity. Rats housed in metabolism cages, were given orally 20 mg nicotinamide and the N-methylnicotinamide (MNA) content in a 24-hour urine col-

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[1] The vitamin mixture provided the following/kg of diet: (in milligrams) thiamine·HCl, 2; riboflavin, 2; pyridoxine, 2.5; Ca pantothenate, 20; niacin, 50; inositol, 100; *p*-aminobenzoic acid, 250; folic acid, 0.20; choline·HCl, 1000; vitamin K, 2.1. Two drops of vitamin A, D and E concentrate were fed to each rat once a week.

lection was determined by a fluorimetric method (4). Urine samples were treated with urea, methyl-ethyl ketone, 40% NaOH and 5 N HCl. After heating at 75° for 3 minutes the fluorescence of the condensation product between MNA and methyl-ethyl ketone was estimated in a Turner fluorometer.

At the end of the experiment (60 days) 25 rats of each group were decapitated and the livers removed as rapidly as possible, wiped free of blood and used for biotin and vitamin B_{12} determinations and for assays of the enzymes.

Biotin was assayed microbiologically on samples of liver homogenate autoclaved with 5 N H_2SO_4 at 121° for one hour. *Lactobacillus arabinosus* 17/5 ATCC 8014 was used as the test microorganism with biotin assay medium.[2] The microbial growth was determined by titrating the lactic acid formed with 0.1 N NaOH after 72 hours' incubation at 30°.

The vitamin B_{12} was also assayed microbiologically on samples of liver homogenate incubated with papain at 60° for one hour. The total vitamin B_{12} activity and the alkali-stable activity were estimated by the USP XV method using *Lactobacillus leichmannii* ATCC 7830 and vitamin B_{12} assay medium.[3]

For assaying betaine-homocysteine transmethylase activity volumes of 20% liver homogenate were incubated with betaine hydrochloride, DL-homocysteine and 0.039 M potassium phosphate buffer, pH 7.4, in a Dubnoff apparatus at 37° for 3 hours under nitrogen (5,6). The reaction was stopped by adding trichloroacetic acid and the methionine present in the filtrate (suitably diluted) was estimated microbiologically using *Lactobacillus mesenteroides* P 60 ATCC 8042 with methionine assay medium.[4] After 72 hours' incubation at 37° the microbial growth was determined by titrating the lactic acid formed with 0.1 N NaOH. For assaying free methionine already present in the liver, tissue samples were treated, after heating at 100° for 2 minutes and homogenizing, with 10% sodium tungstate and 0.6 N H_2SO_4. Methionine was determined on the supernatant after centrifugation.

Ornithine-carbamyl transferase activity was assayed by a spectrophotometric method (7). Volumes of liver homogenate were incubated with suitable quantities of ornithine and carbamylphosphate in arsenate buffer, pH 7.1, at 37° for 24 hours; the reaction was stopped by adding 5 N perchloric acid. The supernatant fraction, after centrifugation, was incubated with 0.01 N HCl in a Conway micro-diffusion unit at 37° for 150 minutes. The ammonia formed was determined by Nessler's reaction in a Beckman spectrophotometer at 416 $m\mu$.

Aspartate-carbamyl transferase activity was estimated by the production of carbamylaspartate resulting from the incubation of volumes of the supernatant, obtained after centrifugation of liver homogenate in 1.15% KCl at 15,000 × *g* for 15 minutes, with carbamylphosphate and L-aspartic acid in 0.2 M Tris buffer, pH 9.2, at 37° for 30 minutes. Following this, enzymatic activity was stopped by adding 2 N perchloric acid (8). The carbamylaspartate concentration was determined by a modified method of Koritz and Cohen (9) in a Beckman spectrophotometer at 550 $m\mu$. Protein was assayed by the colorimetric procedure of Lowry et al. (10).

All the results were analyzed for statistical significance by calculating Fisher's *t* test; a difference between 2 mean values was regarded as significant when *P* was no greater than 0.05.

RESULTS

After 60 days, the animals receiving the biotin-free diet showed the characteristic symptoms of deficiency consisting of loss of hair, "spectacle eyes" and retarded growth, whereas those fed the vitamin B_{12}-supplemented diet did not develop specific symptoms of biotin deficiency. Moreover the data of table 1 show a markedly higher growth rate of the vitamin B_{12}-treated rats (group 3) than that of the biotin-deficient rats (group 1) ($P < 0.001$), although slightly lower than that of the animals given biotin (group 2). The rats treated with both biotin and vitamin B_{12} showed a

[2] Bacto-Biotin Assay Medium (B419), Difco Laboratories, Inc., Detroit.
[3] Bacto B_{12} Assay Medium USP (B547), Difco Laboratories, Inc., Detroit.
[4] Bacto Methionine Assay Medium (B423), Difco Laboratories, Inc.

TABLE 1

Effect of the administration of vitamin B_{12} and biotin on body weight gain of biotin-deficient rats

Group [1]	Supplement to diet	Initial wt	Final wt (60 days)	Wt gain
		g	*g*	*g*
		Experiment 1		
1	None (8) [2]	45(40–48) [3]	102(122–137)	57 ± 6.90 [4]
2	Biotin (8)	45(41–48)	207(182–254)	162 ± 5.36
3	Vitamin B_{12} (8)	45(40–47)	169(151–192)	124 ± 4.51
		Experiment 2		
1	None (8)	65(55–72)	163(132–195)	98 ± 9.74
2	Biotin (8)	65(54–70)	254(238–271)	189 ± 5.14
3	Vitamin B_{12} (8)	65(56–71)	238(190–260)	173 ± 10.34
		Experiment 3		
1	None (15)	57(50–68)	161(126–200)	104 ± 12.27
2	Biotin (15)	58(52–68)	253(198–295)	195 ± 9.72
3	Vitamin B_{12} (15)	58(50–69)	234(175–290)	176 ± 10.06
4	Biotin + vitamin B_{12} (15)	60(50–69)	259(169–324)	197 ± 13.90

[1] Group 1, basal diet containing 11% raw dried egg white; group 2, basal diet containing 11% autoclaved egg white + biotin (100 μg/kg); group 3, basal diet containing 11% raw dried egg white + vitamin B_{12} (100 μg/kg); group 4, basal diet containing 11% autoclaved egg white + biotin (100 μg/kg) + vitamin B_{12} (100 μg/kg).
[2] Figures in parentheses indicate number of animals.
[3] Means and range of individual values.
[4] Means ± SE of mean.

TABLE 2

Reciprocal effects of the administration of vitamin B_{12} and biotin on the liver levels of the vitamins

Group [1]	Supplement to diet	Liver biotin	Liver vitamin B_{12}
		mμg/g fresh tissue	*mμg/g fresh tissue*
1	None	196 ± 60.42 [2]	73.7 ± 5.74
2	Biotin, 100 μg/kg	1542 ± 246.58	52.9 ± 5.42
3	Vitamin B_{12}, 100μg/kg	1013 ± 75.95	166.9 ± 8.19

[1] Five rats/group.
[2] All results are expressed as their averages ± SE mean.

TABLE 3

Effect of the administration of biotin and vitamin B_{12} on methylation in vivo *and* in vitro

Group [1]	Supplement to diet	Betaine-homocysteine transmethylase activity	Methylation capacity in vivo
		μg methionine/hr/g fresh tissue	*mg MNA/100 g body wt/24-hr urine*
1	None	484 ± 49.1 [2]	1.845 ± 0.261
2	Biotin, 100 μg/kg	878 ± 59.2	3.251 ± 0.132
3	Vitamin B_{12}, 100 μg/kg	930 ± 61.4	3.612 ± 0.317

[1] Eight rats/group.
[2] All results are expressed as their averages ± SE mean.

growth rate similar to that of the animals given biotin alone.

The data in table 2 indicate an increased level of biotin in the liver not only in the rats receiving biotin but also in those receiving vitamin B_{12}. In both cases the increase is highly significant in relation to the deficient animals ($P < 0.01$). On the other hand biotin administration caused a small but significant decrease in the vitamin B_{12} hepatic level ($P < 0.05$).

Methylation, in vivo, expressed as urinary elimination of N-methylnicotinamide after nicotinamide administration, was influenced not only by vitamin B_{12} but also by biotin (table 3). N-methylnicotinamide excreted in 24 hours by rats receiving biotin was greater than in the deficient ani-

TABLE 4

Effect of the administration of vitamin B_{12} and biotin on aspartate-carbamyl transferase and ornithine-carbamyl transferase activities

Group [1]	Supplement to diet	Aspartate-carbamyl transferase activity	Ornithine-carbamyl transferase activity
		μmoles carbamylaspartate/g fresh tissue	*mmoles NH_3/g fresh tissue*
1	None	98.8 ± 3.41 [2]	6.27 ± 0.38
2	Biotin, 100 μg/kg	78.1 ± 6.70	7.55 ± 0.36
3	Vitamin B_{12}, 100 μg/kg	74.7 ± 4.24	8.28 ± 0.62

[1] Seven rats/group.
[2] All results are expressed as averages ± SE of mean.

mals ($P < 0.001$). Betaine-homocysteine transmethylase activity was also markedly influenced by biotin administration. In fact the methionine formed from homocysteine and betaine in the liver homogenate of rats receiving biotin was higher than that in the deficient animals and slightly lower than that in rats given vitamin B_{12}.

Finally the data concerning carbamyl transferase activities (table 4) show that: 1) biotin and vitamin B_{12} exert a similar effect on aspartate-carbamyl transferase; both vitamins tend to lower the enzyme activity which is high in biotin deficiency ($P < 0.05$); and 2) both biotin and vitamin B_{12} cause a small but significant increase in ornithine-carbamyl transferase activity ($P < 0.05$).

No significant difference in liver protein content was observed among the groups.

DISCUSSION

The administration of vitamin B_{12} exerts a favorable effect on biotin-deficient animals which results both in better growth and general state of health, and in a delay of the development of the deficiency symptoms. The fact that the addition of vitamin B_{12} to the diet supplemented with biotin gave a growth response similar to that obtained with the supplementation of biotin alone, should be interpreted as evidence of the effect of vitamin B_{12} in alleviating symptoms of biotin deficiency and not as a criterion for deficiency of vitamin B_{12}. This "biotin-sparing" action exerted by vitamin B_{12} on the growth and the delayed development of the deficiency symptoms in the rat may be related to the similar effects these 2 vitamins have on the metabolic reactions studied.

Effect of vitamin B_{12} and biotin on methylation. The role of vitamin B_{12} in methylation in vivo and in vitro has been demonstrated in many previous studies. Oginsky (11), Dietrich et al. (12), Fatterpaker et al. (13), Marchetti et al. (14) and Ranke et al. (15) showed that methionine and creatine formation and the urinary excretion of N-methylnicotinamide decrease markedly in vitamin B_{12} deficiency. Similarly vitamin B_{12} could replace, at least in part, the choline and methionine requirement of the chick, rat and pig. The detailed mechanism of vitamin activity is not yet clear. According to Arnstein (16), vitamin B_{12} has no effect on transmethylation but is involved in the synthesis of labile methyl groups from more highly oxidized precursors such as the α-carbon of glycine and the β-carbon of serine. Kisliuk (17) and Takeyama et al. (18) have indicated that an enzyme containing a derivative of vitamin B_{12} as a prosthetic group is required in the synthesis of the methyl group of methionine. According to Guest et al. (19) a "methylcobalamin" in a transient state appears to be the prosthetic group of the vitamin B_{12}-enzyme in the vitamin B_{12}-dependent pathway for the methylation of homocysteine.

Our data, in addition to confirming this positive effect of vitamin B_{12}, suggest that biotin also may influence these enzymatic activities. A possible explanation of the participation of biotin in these metabolic reactions may result from the investigations of Luckey et al. (20) and Noronha et al. (21). These authors observed that biotin administration to rats fed a biotin- and folic acid (PGA)-free diet caused increased excretion of folic acid, and almost complete restoration of PGA and citrovorum factor liver levels. Therefore it is

suggested that biotin administration may influence PGA synthesis by intestinal microorganisms.

The role of PGA or, better, of folate coenzymes in the metabolism of the one-carbon unit is well-known: the hydroxymethyl groups involved in serine-glycine interconversion and in the biosynthesis of methyl groups of thymine, methionine and choline are transferred by tetrahydrofolic acid (THFA).

Sakami and Ukstins (22) and, more recently, Larrabee et al. (23) have indicated that THFA is involved in methionine synthesis as a precursor of 5-methyl THFA. It is formed from the reaction of 5, 10-methylene THFA with DPNH in the presence of methylene reductase and converted to methionine and THFA by an enzymatic system consisting of DPNH, FAD, ATP, Mg^{++}, homocysteine and a vitamin B_{12}-enzyme.

Biotin activity in methylation may be explained through its action on the storage of folate coenzymes.

Effect of vitamin B_{12} and biotin on carbamyl transferase activities. Until now the reactions involving the carbamyl-phosphate transfer have been studied with respect to their possible biotin dependence. The results obtained thus far on the effect of biotin on carbamyl-phosphate transferase are controversial. In enzyme preparations from *Streptococcus lactis* and *L. arabinosus* biotin deficiency was shown to reduce markedly the carbamylation of ornithine to citrulline and of aspartate to N-carbamylaspartate, indicating a biotin role in these reactions (24, 25).

In no instance, however, did the in vitro addition of free or bound biotin to biotin-deficient rat liver homogenates or to cell-free extracts of *S. lactis* deprived of biotin, restore the normal rate of synthesis of citrulline from ornithine. The rate is restored only when biotin is introduced into the culture medium of the microorganism or in the diet of the rat (26).

These data suggest that biotin exerts an indirect effect on the synthesis of the enzyme proteins and it is not a coenzyme component of the enzyme. This is in accordance with the statement that highly purified preparations of ornithine-citrulline transferase of *S. lactis* contain only 1 part of biotin in 5,000,000 parts of enzyme (27).

Our data show the effect of biotin on these transcarbamylase activities also in the rat: increase of ornithine-carbamyl transferase and decrease of aspartate-carbamyl transferase.

The difference between the deficient and biotin-treated animals are significant although not highly so, in contrast with the data obtained by Mistry and Grillo (28) in liver mitochondria. Vitamin B_{12} has the same effect as biotin. Such an effect on carbamyltransferase activity may be explained by the fact that the vitamin B_{12} administration increases markedly the biotin hepatic level. Therefore the effect on biotin deficiency might result in an increased biotin synthesis by intestinal microorganisms and, as a consequence, in an improved tissue level of the vitamin. On the other hand, this fact does not preclude the possibility vitamin B_{12} may act also in a different way replacing biotin in these metabolic reactions, all the more so as biotin appears to have an indirect role in apoenzyme synthesis.

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Utilization of Co^{58}-labeled Vitamin B_{12} by Biotin-deficient Rats

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ABSTRACT The metabolic pattern of vitamin B_{12} was studied in biotin-deficient rats. The livers of biotin-deficient rats had a significantly higher vitamin B_{12} content than those the biotin-treated animals. Absorption and excretion of orally administered Co^{58}-vitamin B_{12} were not impaired in biotin-deficient rats, whereas the uptake of radioactivity by major organs was significantly higher. No difference in urine and fecal radioactivity was noted in biotin-deficient rats when Co^{58}-vitamin B_{12} was administered by injection. In these animals the retention of the radiovitamin by the organs was noticeably increased.

Studies of vitamin B_{12} have been carried out on the effect of deficiency of other vitamins on the absorption, retention and distribution of vitamin B_{12}. In particular, pyridoxine deficiency resulted in an impaired absorption of Co^{60}-labeled vitamin B_{12}. This impairment can be fully corrected by treatment with pyridoxine (1).

Deficiency of vitamin C resulted in an increase of hepatic uptake of radioactivity after oral or parenteral intake of Co^{60}-labeled vitamin B_{12} (2).

Deficiencies of thiamine, riboflavin or pantothenic acid in rats appeared to have no influence on the absorption of radiovitamin B_{12} (1). However, recently Okuda et al. (3) reported that radioactive vitamin B_{12}, given either orally or by injection, was retained more in the liver in the pantothenic acid-deficient rats than in the control animals given the pantothenic acid supplement.

There are no data in the literature concerning the effect of biotin deficiency on the absorption and utilization of vitamin B_{12}. On the other hand, interrelationships between biotin and vitamin B_{12} have been observed recently (4). The present study, therefore, was undertaken to investigate the vitamin B_{12} levels in liver, as well as the pattern of absorption, retention, distribution among major organs, and the excretion of Co^{58}-labeled vitamin B_{12} in biotin-deficient rats.

EXPERIMENTAL

Animals and diets. Weanling male rats of the Wistar strain were divided into 2 groups and housed in cages with screen bottoms. The rats of group 1 were fed a biotin-free diet, having the following composition: (in per cent) vitamin-free casein, 20; sucrose, 59; salt mixture IV (5), 4; groundnut oil, 5; raw dried egg white, 11; and vitamin mixture,[1] 1. The rats of group 2 were fed the same diet, but the raw dried egg white was replaced by an egg white autoclaved at 120° for 15 minutes and dried. Biotin (100 μg/kg) was added to the diet of group 2. Feed and water were provided ad libitum.

Determination of microbiological activity of vitamin B_{12} in liver. After feeding the experimental diets for 60 days, 6 rats in each group were killed. The livers were removed immediately, wiped free of blood and used for vitamin B_{12} determination.

The vitamin B_{12} content was estimated by microbiological assay on samples of liver homogenate incubated with papain at 60° for one hour. The total vitamin B_{12} activity and the alkali-stable activity were measured by the USP XV method (6), using *Lactobacillus leichmanni* ATCC 7830 and vitamin B_{12} assay medium.[2]

Measurement of vitamin B_{12} absorption. Co^{58}-vitamin B_{12} with a specific activity of 1 mc/mg was used.[3] After 60

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[1] Each gram of the vitamin mixture contained: (in milligrams) thiamine·HCl, 0.2; riboflavin, 0.2; pyridoxine, 0.25; Ca pantothenate, 2.0; niacin, 5.0; inositol, 10.0; p-aminobenzoic acid, 25.0; folic acid, 0.02; choline·HCl, 100.0; vitamin K, 0.21. Two drops of vitamin A, D and E concentrate were given orally to each rat once a week.

[2] Bacto B_{12} Assay Medium USP (B547), Difco Laboratories, Inc., Detroit.

[3] The Co^{58}-vitamin B_{12} was obtained from the Radiochemical Centre, Amersham, Bucks, England.

days of consuming the experimental diets, 6 animals in each group were given by stomach tube 1 ml of an aqueous solution of Co^{58}-vitamin B_{12} containing 0.20 μg. The animals were transferred to individual metabolism cages for collection of fecal and urine specimens.

Four days after administration of radioactive vitamin B_{12}, the rats were killed. Urine, feces, liver, kidneys, gastrointestinal tract, heart and spleen were saved for Co^{58} determination. The gastrointestinal tract, from the cardia through the end of colon, was stripped free of supporting tissue, immediately opened lengthwise and washed. The various organs were dissolved in alkali (30% KOH), feces were softened with water and homogenized with concentrated H_2SO_4, and the urine was concentrated over steam baths.

Four milliliters of all radioactive samples were counted using standards of comparable geometry in a low background gamma scintillation counter with a thallium-activated sodium iodide crystal installed in a well-type sample-holder (Tracerlab Model SC 57/A).

Measurement of excretion and retention of vitamin B_{12}. After 60 days of receiving the experimental diets, 6 animals in each group were injected subcutaneously with 0.20 μg of Co^{58}-vitamin B_{12} in a saline solution and housed in individual metabolic cages. After 4 days, the rats were killed and the radioactivity in urine and feces, as well as in various organs, was determined.

All of the results were analyzed for statistical significance by Fisher's t test, a difference between 2 mean values being regarded as significant when P was no greater than 0.05.

RESULTS

Liver biological activity of vitamin B^{12}. The results tabulated in table 1 demonstrate that the rats receiving a biotin-free diet had a significantly higher vitamin B^{12} content in liver ($P < 0.05$) than the biotin-treated control animals.

Absorption and organ uptake of orally administered Co^{58}-vitamin B_{12}. The radioactivity present in liver, kidneys, gastrointestinal tract, heart and spleen is expressed as the percentage of total oral dose as well as the percentage of dose per gram of wet tissue (table 2).

The uptake of radioactivity was higher in the kidneys ($P < 0.05$) and in the gastrointestinal tract ($P < 0.02$) of biotin-deficient rats than that in the same organs of rats that had received the biotin-supplemented diet, when expressed as a percentage of total dose. No significant

TABLE 1

Effect of biotin deficiency on vitamin B_{12} content of rat liver

Group	Treatment	Final wt	Liver vitamin B_{12}
		g	*mμg/g fresh tissue*
1	Biotin-deficient	155 ± 13.2 [1] (18) [2]	81.1 ± 6.50 (6)
2	Biotin-treated	267 ± 21.6 (18)	47.9 ± 6.22 (6)

[1] Mean ± SE of mean.
[2] Numbers in parentheses indicate number of rats.

TABLE 2

Effect of biotin deficiency on organ distribution of orally administered Co^{58}-vitamin B_{12} in the rat

Tissue	Recovery of administered Co^{58}-vitamin B_{12}			
	Biotin-deficient rats (6) [1]		Biotin-treated rats (6)	
	% of total dose	*% of dose/g tissue*	*% of total dose*	*% of dose/g tissue*
Liver	4.75 ± 0.63 [2]	0.85 ± 0.13	4.48 ± 0.45	0.48 ± 0.04
Kidney	6.53 ± 0.54	3.49 ± 0.41	4.66 ± 0.52	2.01 ± 0.20
Gastrointestinal tract	7.60 ± 0.70	1.00 ± 0.08	5.36 ± 0.28	0.63 ± 0.04
Spleen	0.38 ± 0.04	0.67 ± 0.04	0.61 ± 0.04	0.61 ± 0.04
Heart	0.31 ± 0.02	0.65 ± 0.10	0.38 ± 0.09	0.44 ± 0.09

[1] Numbers in parentheses indicate number of rats.
[2] Mean ± SE of mean.

variation was noted in the uptake of radioactivity in the liver, in the spleen and in the heart.

The uptake of radioactivity was significantly increased, not only in the kidneys ($P < 0.01$) and in the gastrointestinal tract ($P < 0.001$), but also in the liver ($P < 0.01$) and heart ($P < 0.05$) of biotin-deficient rats as compared with biotin-treated animals, when the results were expressed as percentage of dose per gram of wet tissue.

The difference between the test dose and fecal recovery of radioactivity was taken as the absorption (table 3). No significant difference in absorption and urinary excretion of radioactive vitamin was found between deficient and biotin-supplemented rats.

Organ uptake and excretion of injected Co^{58}-vitamin B_{12}. Data on the recovery of radioactivity in the various organs are shown in table 4.

The liver, kidneys and gastrointestinal tract of the deficient rats retained more radioactivity than those of the biotin-treated animals on the basis of percentage of total dose ($P < 0.001$; $P < 0.01$; $P < 0.01$, respectively), whereas no difference was noted in the radioactivity of the heart and spleen. On the contrary, if the results of radioactivity were expressed as percentage of dose per gram of wet tissue, a significant increase was observed in all the organs of the biotin-deficient rats as compared with the control animals receiving biotin ($P < 0.001$).

No significant difference in the radioactivity of urine and feces was observed in the rats fed the biotin-free diet as compared with the animals receiving the diet supplemented with biotin (table 5).

TABLE 3

Effect of biotin deficiency on absorption of orally administered Co^{58}-vitamin B_{12} in the rat

Group	Treatment	Radioactivity		
		Urine	Feces	Absorption [1]
		% of total dose	*% of total dose*	*% of total dose*
1	Biotin-deficient (6) [2]	1.08 ± 0.08 [3]	61.6 ± 1.73	38.4 ± 1.48
2	Biotin-treated (6)	1.54 ± 0.38	59.4 ± 6.19	40.6 ± 5.27

[1] Calculated by subtracting fecal radioactivity from oral dose.
[2] Numbers in parentheses indicate number of rats.
[3] Mean ± SE of mean.

TABLE 4

Effect of biotin deficiency on retention of injected Co^{58}-vitamin B_{12} in the rat

Tissue	Recovery of administered Co^{58}-vitamin B_{12}			
	Biotin-deficient rats (6) [1]		Biotin-treated rats (6)	
	% of total dose	*% of dose/g tissue*	*% of total dose*	*% of dose/g tissue*
Liver	9.99 ± 0.62 [2]	1.46 ± 0.08	6.57 ± 0.16	0.60 ± 0.04
Kidney	15.60 ± 0.86	9.38 ± 0.65	10.05 ± 0.97	3.91 ± 0.33
Gastrointestinal tract	11.99 ± 0.50	1.69 ± 0.11	8.51 ± 0.88	1.03 ± 0.12
Spleen	0.93 ± 0.01	1.47 ± 0.06	1.05 ± 0.02	0.98 ± 0.05
Heart	0.57 ± 0.01	1.03 ± 0.05	0.63 ± 0.01	0.69 ± 0.01

[1] Numbers in parentheses indicate number of rats.
[2] Mean ± SE of mean.

TABLE 5

Effect of biotin deficiency on excretion of injected Co^{58}-vitamin B_{12} in the rat

Group	Treatment	Radioactivity		
		Urine	Feces	Total in 4 days
		% of total dose	*% of total dose*	*% of total dose*
1	Biotin-deficient (6) [1]	10.49 ± 0.38 [2]	9.61 ± 0.80	20.10 ± 0.78
2	Biotin-treated (6)	8.46 ± 0.06	8.13 ± 1.86	16.59 ± 1.72

[1] Numbers in parentheses indicate number of rats.
[2] Mean ± SE of mean.

DISCUSSION

Results of the present studies indicate that the biotin-treated rats retained less of the radioactive vitamin B^{12} administered, either orally or subcutaneously, in the organs studied than did the biotin-deficient animals.

In this respect the microbiological activity of vitamin B_{12} in the liver of the biotin-treated rats was also lower than that of the deficient animals.

Since no significant variation was noted in the absorption nor in the excretion of Co^{58}-vitamin B_{12} between biotin-treated and deficient groups, the observed changes in the retention and distribution of the radioactive vitamin in the organs must be dependent upon other mechanisms.

It has been shown previously that the administration of vitamin B_{12} (100 μg/kg of diet) to rats maintained with a biotin-free diet appears to influence biotin-dependent enzymatic activities and exerts a favorable effect on the growth rate and on the delay of deficiency symptoms (4). These observations appear to be contradictory since biotin-deficient rats have high microbiological activity of vitamin B_{12} in the liver; nevertheless they require more vitamin B^{12}, which is suggested also by the higher retention of radioactivity in storage organs, when Co^{58}-vitamin B_{12} is given either by injection or orally.

However, if utilization of vitamin B_{12} in biotin deficiency is interfered with, or the organ vitamin B_{12} is not metabolically available, administration of a large quantity of vitamin B_{12} could produce a beneficial effect. This hypothesis could explain both the higher level of vitamin B_{12} in the liver, and the retention of radioactivity in the biotin-deficient rats.

Although additional data will be necessary before any interpretation can be drawn with certainty, the results of the experiments presented here suggest that in biotin-deficient rats the utilization of vitamin B_{12} is impaired.

Therefore, these results confirm a relationship of biotin with vitamin B_{12} as first observed in our previous studies (4).

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Effect of Dietary Vitamin K on the Nutritional Status of the Rhesus Monkey [1]

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ABSTRACT As a result of dietary vitamin K deficiency, male *Macaca mulatta* monkeys had prolonged prothrombin times which reversed spontaneously or could be decreased sharply by the administration of small quantities of vitamin K_1. This pattern of prolongation and spontaneous reversal was also observed when neomycin sulfate and oxytetracycline were administered. The feeding of irradiated beef diets caused prolongation of the prothrombin time of only one pre-adolescent monkey, although the irradiated beef diets did not contain sufficient vitamin K to maintain rats, even when coprophagy was permitted.

The need for exogenous vitamin K by the human infant (1), the growing male rat (2), the chick (3), the baby pig (4), and the cholecystnephrostomized pup (5) has been established. In monkeys, Metta and Gopalan [4] observed normal plasma prothrombin times when "vitamin-free" casein diets containing little vitamin K activity were fed, even if synthesis of the vitamin by intestinal microflora was inhibited by the administration of sulfasuxidine and aureomycin. Since diets which provide soy protein as the protein source appear to be more satisfactory for the production of vitamin K deficiency than those which use "vitamin-free" casein (3), and since ground beef preserved by γ-irradiation contains vitamin K in amounts inadequate to prevent hemorrhagic diathesis in growing male rats (6), these rations were fed to monkeys with and without the administration of neomycin sulfate and oxytetracycline to produce vitamin K deficiency by nutritional means.

EXPERIMENTAL

All monkeys were housed individually in a temperature- and humidity-controlled laboratory. For approximately one hour daily, they were permitted to exercise freely in this room while their cages were being cleaned and disinfected,[5] and the laboratory floor, walls, and tables scrubbed.

Five monkeys (group 1) were 9 to 15 months of age upon arrival [6] and weighed between 1.0 and 1.8 kg. They were fed a commercial monkey biscuit [7] for the first 15 weeks, and thereafter, beginning with experimental day 1, were given the soy protein, vitamin K-deficient diet (table 1). Between the 149th to 168th day on experiment, monkey no. 2 was given 4 doses of vitamin K_1, 100, 50, 25, and 10 μg, respectively. On the 198th through to the 205th day, 150 mg of neomycin sulfate [8] per kg of body weight were administered orally to monkey no. 1 daily. On day 214, oral treatment with neomycin sulfate was resumed and oxytetracycline hydrochloride [9] was given concurrently. On day 214, monkey no. 2 was started on oral neomycin sulfate. The dose schedules for these broad spectrum antibiotics varied and are shown in table 2. Approximately midway through the antibiotic supplementation period, the vitamin and mineral components of the diets were increased 2- and 1.5-fold, respectively, to counteract possible defi-

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[2] Present address: Winthrop College, The South Carolina College for Women, Rock Hill, South Carolina.

[3] Present address: Department of Biochemistry, Indian Institute of Science, Bangalore 12, India.

[4] Metta, V. C., and C. Gopalan 1962 A study of the vitamin K requirement of the monkey. Federation Proc., 21: 387 (abstract).

[5] Wescodyne, Wescodyne Disinfecting Company, Long Island City, New York.

[6] Shamrock Farms, Inc., Middletown, New York.

[7] Ralston Purina Company, St. Louis.

[8] Neomycin sulfate (Biosol) was generously supplied by the Upjohn Company, Kalamazoo, Michigan, through the courtesy of Dr. Jack L. Davidson.

[9] Oxytetracycline hydrochloride was generously supplied by Pfizer and Company, New York, through the courtesy of Dr. J. H. Hare.

TABLE 1

Composition of diets fed Rhesus monkeys [1]

Soy protein diet			Irradiated beef diets	
			Group 1	Group 2
	g		*g*	*g*
Soy protein [2]	20.0	Beef [3]	85.0	100.0
DL-Methionine [4]	0.5	Vitaminized sucrose [5]	10.0	12.0
Glyceryl trioleate	3.0	Starch	10.0	20.0
Methyl linoleate	0.6	Sucrose	36.0	35.0
Salts 446 [6,7]	4.0	Glycerol trioleate	1.0	7.0
Glucose monohydrate	71.7	Methyl linoleate	0.3	0.4
Vitamins [6,8]	0.2	Salts 446	2.0	2.0

[1] 20 kcal/g of protein.
[2] ADM C-1 Assay Protein, Archer-Daniel-Midland Company, Cincinnati, Ohio
[3] Beef fed to group 1 contained 21.9% protein and 8% fat; beef fed to group 2 was obtained from a bull, was very well trimmed, and contained 21.5% protein and 1% fat.
[4] Generously supplied by Dow Chemical Company, Midland, Michigan.
[5] Mg/100 g of sucrose: thiamine·HCl, 3.5; riboflavin, 3.5; pyridoxine·HCl, 3.5; Ca pantothenate, 20; niacin, 25; vitamin B_{12}, 1; biotin, 0.2; folic acid, 2.0; inositol, 200; *p*-aminobenzoic acid, 300; vitamin E succinate, 73; choline chloride, 1000; ascorbic acid, 500; vitamin A (250,000 IU/g), 120; vitamin D (5,000 IU/g), 300.
[6] The concentrations of minerals in the diet were increased 1.5-fold and of vitamins twofold, whenever antibiotic dosages greater than 200 mg/kg/body wt/day were administered.
[7] Mameesh, M. S., and B. C. Johnson 1958 J. Nutrition, 65: 161.
[8] Mg/100 g of diet: thiamine·HCl, 0.35; riboflavin, 0.35; pyridoxine·HCl, 0.35; Ca pantothenate, 2.0; niacin, 2.5; cyanocobalamin, 0.1; biotin, 0.02; folic acid, 0.2; *p*-aminobenzoic acid, 30.0; vitamin E succinate, 7.3: (the above vitamins were generously supplied by Merck, Sharp and Dohme, Rahway, New Jersey); inositol, 20.0; choline chloride, 100. IU/100 g of diet: vitamin A, 3,000 and vitamin D, 500, generously supplied by Endo Products Company, Richmond Hill, New York; 50 mg of ascorbic acid given daily in drinking water.

TABLE 2

Mean prothrombin times for monkeys (group 1) fed soy protein rations

No. animals	Treatment [1]	Days	No. blood samples/ animal	Prothrombin time
				sec
1	None	197	15	18.0 (13.5–29.5)
1	N, 150 mg/kg	8	3	23.0 (20.0–24.8)
1	None	8	—	—
1	N, 150 mg/kg; 0, 20 mg/kg	32	7	24.1 (18.0–33.2)
1	N, 225 mg/kg; 0, 30 mg/kg	6	1	17.7
1	N, 225 mg/kg; 0, 60 mg/kg	10	4	22.8 (21.5–25.2)
1	N, 300 mg/kg; 0, 100 mg/kg	6	3	24.0 (21.5–28.5)
1	Vitamin K_4 [2]	2	5	25.4 first value 16.2 last value
2	None	148	7	21.3 (15.5–31.7)
2	Vitamin K_4 [3]	22	4	27.8 (22.8–43.0)
			4	14.8 (14.0–15.3)
2	None	43	5	19.7 (18.3–21.4)
2	N, 150 mg/kg	32	7	23.8 (16.0–29.1)
2	N, 300 mg/kg	16	5	23.9 (20.7–26.0)
2	N, 400 mg/kg	6	4	28.0 (17.0–35.5)
2	Vitamin K_4 [4]	2	5	33.0 first value 15.0 last value
3	None	268	34	18.8 (12.8–26.0)
3	Vitamin K_4 [5]	1	2	21.0 first value 13.4 last value

[1] N, neomycin sulfate; O, oxytetracycline hydrochloride.
[2] 0.104 μg/kg in 2 injections over 30 hours.
[3] Four injections: 100, 50, 25, and 10 μg over 22 days. Larger prothrombin time is the average peak at which time vitamin K_4 was given. Smaller value is the average depression after vitamin K_4 injection.
[4] 0.105 μg/kg in 3 injections over 28 hours.
[5] 0.20 μg/kg.

ciencies other than vitamin K which might occur as a result of concomitant diarrhea. Menadione disodium phosphate (vitamin K_4)[10] was given at the end of the antibiotic period.

In a preliminary study on the influence of the feeding of irradiated beef, 3 of the original 5 monkeys which had been given vitamin K after treatment with antibiotics were fed a ground beef diet, days 269 to 293. For the following 70 days, they were fed a similar diet (table 1), but the beef, which had been trimmed to contain 8% fat, ground, canned and frozen, was γ-irradiated at 5.6 megarads. This irradiated beef was stored frozen until used.

The second group (2) of monkeys was used only for irradiated beef studies. These 6 male *Macaca mulatta* monkeys weighed 1.5 to 1.9 kg upon arrival [11] and were fed biscuits and fruit for 4 days. Two animals were then given non-irradiated beef diets, and 4 irradiated beef rations ad libitum (table 1). For this experiment, deboned bull meat was obtained and trimmed of all visible fat. It contained only about 1% fat when ground, canned and frozen. Part of this beef was γ-irradiated at 5.6 megarads; the other remained in frozen storage until used in the diets. The monkeys received these beef diets for 70 days, following which each animal was injected intramuscularly with 100 μg of vitamin K_4.

Collection of blood samples and measurement of prothrombin time have been described elsewhere (4).

RESULTS

Group 1. Two of the 5 monkeys began to excrete blood and died after 2 months of the soy protein, vitamin K-deficient diet. Postmortem examination [12] failed to reveal the presence of pathogens as the cause of death; however, the hemorrhages were not as extensive as those observed in male rats dying of vitamin K deficiency.

Slight prolongation of mean prothrombin times occurred when the soy protein diet was fed to the remaining 3 monkeys and became more marked with antibiotic treatment (table 2). The elevated values could be counteracted by administration of vitamin K_4. Progressive changes in prothrombin times for 2 monkeys given antibiotics are shown in figure 1.

The monkeys which received the large doses of antibiotics had severe diarrhea, hypersensitivity and anorexia, and were very listless. A control, non-irradiated ground beef diet was given on day 270, and it was several days before the monkeys became frisky again and gained weight. When the feeding of irradiated beef diets was begun (day 294), the prothrombin times of the monkeys were less than 20 seconds and did not change during the 70 days of feeding, nor after 10 subsequent days when fed non-irradiated beef diets. These data are summarized in table 3.

The monkeys were killed and autopsied [13] after completion of the experimental period and were judged healthy. The results from a control study on weanling male rats fed the same diets are shown in table 4. Two of the rats fed the irradiated beef diet, and one of the rats given the soy protein, vitamin K-deficient diet (2) died.

Group 2. Marked prolongation of prothrombin time did not occur when 4 preadolescent monkeys were fed irradiated beef diets which contained 1% natural fat. The longest time was 24.4 seconds; the highest value for a control was 19.5 seconds. One hundred micrograms of vitamin K were administered to each of the animals on the 70th experimental day, and all monkeys had prothrombin times between 13 and 15 seconds 3 days later. Weanling rats were fed beef diets (1% natural fat) containing 20 kcal/g of protein, but the ratio of fat to protein was increased to 1.2:1 by the addition of the synthetic fats, glycerol trioleate and methyl linoleate. Six

[10] Herein the term vitamin K_4 refers to the tetrasodium salt of the diphosphate of vitamin K_4, 2-methyl-1,4-naphthohydroquinone.

[11] Asiatic Animal Imports, Inc., San Francisco.

[12] Postmortem examinations were performed under the direction of Dr. E. I. Pilchard, Director of the Illinois State Department of Agriculture Research and Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana. Dr. Pilchard reported: "No pathogenic bacteria could be recovered from the tissues. Tissue sections from the large intestine revealed that the epithelial lining of many of the villi was characterized by a decreased cellularity suggesting atrophy to degenerative change. The vessels within the villi were congested and in some cases, there was a hemorrhagic exudate between the villi. The submucosal lymphoid aggregates appeared hyperplastic; some were quite hemorrhagic with an accumulation of polymorphonuclear leucocytes. In some areas of the mucosa there are lymphocytic aggregates."

[13] Courtesy of Dr. L. E. St. Clair, Professor, College of Veterinary Medicine, University of Illinois, Urbana.

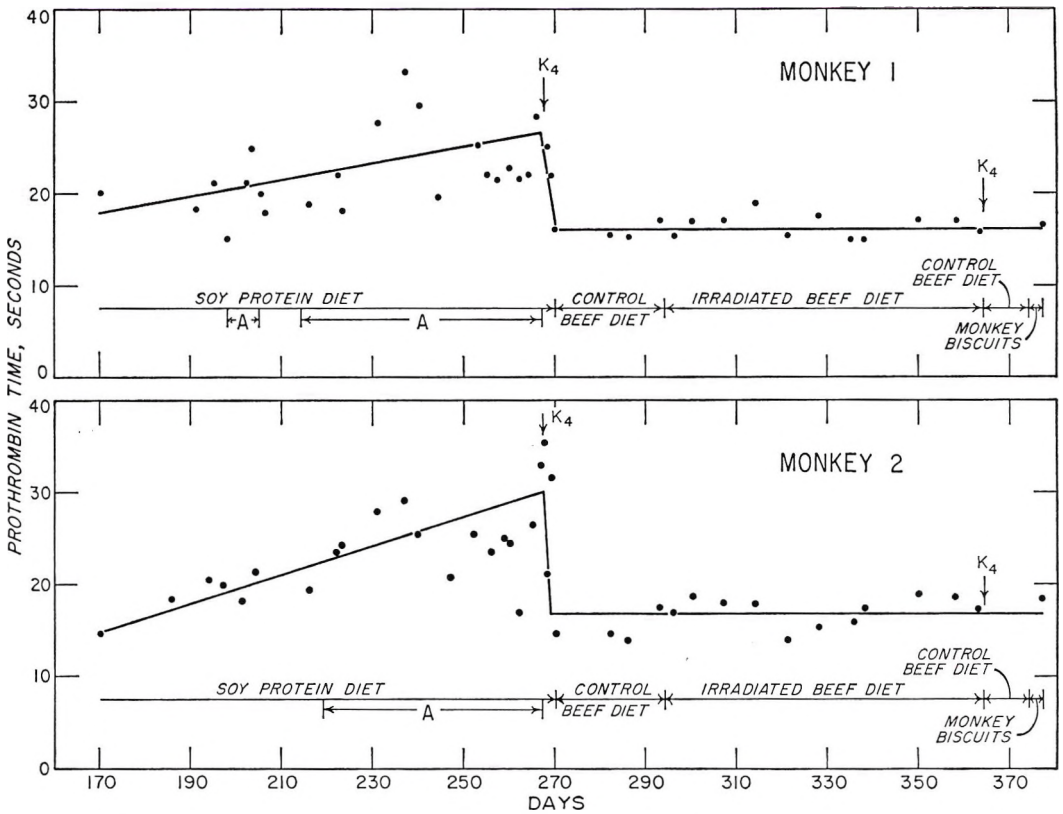

Fig. 1 Progressive changes in prothrombin times for monkeys 1 and 2 (group 1) given antibiotics. Antibiotic doses (A) were not constant and are shown in table 2, as are vitamin K_4 doses.

TABLE 3

Mean prothrombin times for monkeys (group 1) and rats fed γ-irradiated beef

Animal	No.	Diet [1]	Length of feeding	No. blood samples/ animal	Prothrombin time [2]	Deaths
			days		*sec*	
Monkeys	3	Non-irradiated beef	25	4	15.6 (11.5–18.4) [3]	0
	3	Irradiated beef	70	11	17.0 (14.0–19.3)	0
	3	Non-irradiated beef	10	1	17.8 (17.0–18.4)	0
Rats	5	Soy protein plus vitamin K_4 5 μg/day	51	1	14.6 (14.0–15.0)	0
	5	Soy protein	51	1	18.3 (15.0–23.0)	1
	9	Irradiated beef	45–51	1	24.0 (15.0–55.0)	2

[1] Composition of beef diets is shown in table 1. Soy protein diets are taken from Johnson et al. (3).
[2] Excludes values for rats which died.
[3] Numbers in parentheses indicate range.

of 10 rats given the same irradiated beef as the pre-adolescent monkeys, died; internal hemorrhages were evident. All the rats receiving non-irradiated and irradiated beef which contained 15% natural fat survived.

The monkeys in group 2 were also killed and autopsied[14] and were judged healthy.

DISCUSSION

Both soy protein and beef, irradiated and non-irradiated, have been used with purified fats and synthetic ingredients to make diets low in vitamin K. Although no toxic compounds are produced during ir-

[14] See footnote 13.

radiation of beef (3), there is destruction of vitamin K, the residual amount being dependent upon the fat content (i.e., on the vitamin K content) of the beef (table 4) and on the total irradiation dose. Beef irradiated at 5.6 megarads was found to contain approximately one-half of the vitamin K activity of non-irradiated beef, by chick assay (7).

For the rat, a major source of vitamin K is recycled feces. Rats fed irradiated beef —in comparison with those fed soy protein—show signs of vitamin K deficiency more frequently and may not practice coprophagy to as great an extent, perhaps because of distasteful, long-chain carbonyls in their feces (3). When the vitamin K synthesized by intestinal microflora is not available to rats given diets low in vitamin K, i.e., when coprophagy is prevented, or when intestinal synthesis of vitamin K is inhibited by the feeding of sulfa drugs or antibiotics, or both, rats develop hypoprothrombinemia and hemorrhage and die.

Monkeys do not practice coprophagy and thus, must depend upon a dietary intake of vitamin K or upon direct absorption of the vitamin K produced by the intestinal microflora to provide sufficient vitamin K to prevent a deficiency state. Two of 5 monkeys fed a soy protein, vitamin K-deficient diet did not survive and their deaths were preceded by anal bleeding. Vitamin K-deficient rats at death show extensive hemorrhage, particularly in the thoracic cavity and testes; the lesions in the monkeys were much less severe. No deaths, nor even prolonged prothrombin times, occurred when irradiated beef diets were given to monkeys, whereas 6 of 10 rats fed the same diet died within 6 weeks. Thus, it is evident that the monkey is much less susceptible to vitamin K-deficiency than is the male rat.

An average prothrombin value of 13.2 and range of 10.5 to 18.5 seconds have been reported (8) for monkeys, and it is suggested that a prothrombin time of 20 seconds or longer indicates hypoprothrombinemia. When the monkeys were fed irradiated beef diets, mean prothrombin times did not exceed 20 seconds. The monkeys given the soy protein diet showed increases and at times spontaneous decreases of prothrombin time. Addition of antibiotics to the soy protein diet resulted in somewhat more prolonged prothrombin times.

When prolonged prothrombin times were observed in monkeys fed the soy protein diet with or without antibiotics, treatment with vitamin K_1 resulted in sharp decreases. With the highest doses of antibiotics, severe malabsorption was induced, and symptoms of malabsorption syndrome frequently include signs of vitamin K deficiency. Approximately 0.1 μg of vitamin K_1/kg body weight was necessary to decrease the prolonged prothrombin time to within the normal range. Metta and Gopalan [15] noted no changes in prothrombin times of monkeys fed synthetic diets containing 1% sulfasuxidine with a daily supplement of 250 mg. of aureomycin. Their diet contained vitamin-free casein which may furnish more vitamin K than soy protein (3).

[15] See footnote 4.

TABLE 4

Mean prothrombin times for monkeys (group 2) and rats fed γ-irradiated beef

	No. of animals	Diet [1]	Length of feeding	No. blood samples/ animal	Prothrombin time [2]	No. deaths
			days		*sec*	
Monkeys	2	Non-irradiated beef, 1%	70	7	16.2 (12.6–19.5) [3]	0
	4	Irradiated beef, 1%	70	7	16.7 (11.6–24.4)	0
Rats	10	Non-irradiated beef, 1%	42	1	20.7 (15.0–27.0)	0
	10	Irradiated beef, 1%	42	1	17.8 (16.0–23.0)	6
	10	Non-irradiated beef, 15%	42	1	17.2 (16.0–23.0)	0
	10	Irradiated beef, 15%	42	1	16.4 (16.0–17.0)	0

[1] Composition of monkey diet is shown in table 1. For beef 15%, natural fat was added to beef 1% prior to irradiation. The diets prepared for rats all contain fat in the ratio of 1.2:1 to protein, by the addition of methyl linoleate and glyceryl trioleate.
[2] Excludes values for rats which died.
[3] Numbers in parentheses indicate range.

The deaths of 2 monkeys fed vitamin K-low soy protein diets, and prolongation of prothrombin times in other monkeys fed the soy protein diets with and without antibiotics, suggest that it is possible to produce vitamin K deficiency in the *Macaca mulatta* monkey. However, the resistance of the monkey to dietary vitamin K deficiency in comparison with the coprophagy-prevented rat fed the same vitamin K-deficient ration, indicates that the vitamin K synthesized by the intestinal microflora, can be absorbed directly by the monkey, whereas the rat must obtain this vitamin K by coprophagy.

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Metabolism of Ethyl Arachidonate-1-C^{14} in Rats Fed Complete or Fat-free Diets [1]

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ABSTRACT Rats fed complete or fat-free diets absorbed 69 to 77% of a tracer dose of orally administered ethyl arachidonate-1-C^{14} in 6 hours. The arachidonate-1-C^{14} was 95% pure by gas-liquid radiochromatography and was used without further purification. Of the activity absorbed the amount expired as $C^{14}O_2$ was 7% by the animals fed the fat-free diet and 10% by those fed the complete diet and by rats fed the fat-free diet but given acute supplementations of arachidonic acid. A small amount of C^{14}-activity was observed in liver, heart, and testes; the bulk of the radioactivity was in the carcass. About 25% of the administered dose was in carcass water-soluble compounds, extractable with ethyl ether. Livers of fat-deficient rats and those fed the supplement had lower C^{14} content and concentration than controls, but in heart and gonads the concentration of C^{14} was similar in the 3 groups. The major portion of the radioactivity in liver was observed in microsomes and in mitochondria and as phospholipid. Most of the radioactivity was associated with a material which had a retention time (gas-liquid chromatography) similar tc that of arachidonic acid. However, in heart and gonads of fat-deficient animals, a decreased proportion of activity was in arachidonic acid and increased amounts in shorter chain acids. C^{14} was also observed to be associated with materials of retention times greater than that of arachidonic acid, particularly those similar to docosapentaenoic and docosahexaenoic acids.

The essentiality of certain polyunsaturated fatty acids for the rat has been well established. Whether dietary linoleic acid is needed to furnish linoleic acid itself for the animal or as a precursor for arachidonic acid is not known. Partly responsible for our lack of knowledge in this field has been the paucity of information concerning the metabolic fate of arachidonic acid in the animal. Little information is available concerning the biochemistry of this polyunsaturated fatty acid other than serum and tissue concentrations and its biosynthesis from linoleate and acetate as shown by Mead and his co-workers (1, 2). It is apparent that present-day metabolic studies depend largely on the availability of isotopically labeled materials. The acquisition [2] of a small amount of arachidonic acid-1-C^{14} prompted us to undertake the studies reported in this paper of the absorption and metabolism of arachidonic acid in rats fed a normal diet, a fat-free diet, and a fat-free diet plus a limited supplement of non-isotopic arachidonic acid.

EXPERIMENTAL

The ethyl arachidonate-1-C^{14} was 97% pure by gas-liquid chromatography and about 95% pure by gas-liquid radiochromatography. For the latter analysis, 2 methods were used. In method 1 some of the labeled material was mixed with a few milligrams of pure non-radioactive ethyl arachidonate. The specific activity of this mixture was then determined. It was then purified in a preparative model gas-liquid chromatograph, using a 1.3 × 183 cm column packed with ethylene glycol-succinate polyester coated on chromosorb W. The effluent from the column was split, with about 15% supplied to an argon detector and 85% going to a collector composed of a long glass tube packed loosely with defatted cotton. The collected ethyl arachidonate was eluted from this collector and the specific activity determined. It differed from the specific activity of the non-chromatographed sample by about 3%. The second method of checking the radiopurity of the C^{14}-ethyl arachidonate was by means of the gas-liquid radio-

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[2] We are indebted to Hoffmann-LaRoche, Nutley, New Jersey, for the ethyl arachidonate-1-C^{14} and for a generous supply of non-radioactive ethyl arachidonate.

chromatographic method described by Popják[3] and co-workers (3). About 5% of the radioactivity of the injected sample was observed in a peak other than that of ethyl arachidonate, and this was essentially all in a peak corresponding to a trienoic acid of 20-carbon chain length.

All gas-liquid chromatography was carried out with polyester columns (ethylene glycol esterified with either adipic acid or succinic acid). For some of the chromatography 183- or 244-cm coiled columns and a gas density balance detector were used; for other chromatography 244-cm U-shaped columns and an argon ionization detector were used. Monitoring of the radioactivity of the effluent from the gas-liquid chromatograph was done either by the Popják technique indicated previously or by collection of the effluent in anthracene cartridges in a manual collector similar to the automatic collector described by Karmen et al. (4). Standards used for establishing retention times and for quantitative calibration of the argon detector were obtained from the Hormel Institute, Applied Science Corporation, and the National Heart Institute. The efficiency of collection in the anthracene cartridges varied from about 60% (of the amount injected into the column) for methyl laurate to 80% for methyl eicosanoate. Proper corrections of the collected activities were made based on these figures.

Male, weanling rats were fed a fat-free diet containing 20% casein, sucrose, salts, cellulose flour, and adequate quantities of water-soluble and lipid-soluble vitamins.[4] The control rats were pair-fed to the experimental rats and received a diet similar to theirs but containing 20% fat (a partially hydrogenated cottonseed oil)[5] which was substituted for an equal weight of sucrose. The complete diet contained 1.36% by weight of dienoic (linoleic), 0.05% trienoic (linolenic), and no measurable tetraenoic acid. The animals were fed these diets for 8 weeks at which time one-half of the animals fed the fat-deficient diet received a supplement of 300 mg of non-radioactive ethyl arachidonate daily for 2 or 5 days. (Since no differences in intestinal absorption, oxidation to $C^{14}O_2$, or organ distribution of the C^{14}-arachidonate were observed between the animals of the 2-day and 5-day supplementation experiments, for convenience these rats have been treated as one group). All rats were given by stomach tube a tracer dose of carboxyl-labeled C^{14}-ethyl arachidonate in 0.5 ml myristic acid carrier containing 1.8 μc and 0.5 mg arachidonate. Each rat was kept in a metabolic chamber for 6 hours for collection of expired $C^{14}O_2$ and then decapitated. Selected organs were removed, weighed and stored at $-20°$ until analysis was performed. C^{14} activity in expired carbon dioxide was determined by windowless gas-flow counting of $BaC^{14}O_3$ and by liquid scintillation counting of $BaC^{14}O_3$ using Cab-O-Sil.[6] Total fatty acids were obtained from tissues by hydrolysis with potassium hydroxide and extraction with petroleum ether after removal of non-saponifiable material and subsequent acidification. Separation of lipids was made by the silicic acid column chromatography method of Hirsch et al. (6), monitored by thin-layer chromatography. Subcellular liver particles were obtained by homogenization of 0.25 M sucrose and centrifugation at 10,000 $\times$ g (mitochondria) and 100,000 $\times$ g (microsomes) after removal of nuclei and debris at low speeds (600 $\times$ g). C^{14} activity in fatty acid samples was determined in a liquid scintillation spectrometer.

RESULTS

The results of the intestinal absorption of the administered C^{14}-arachidonate and of its oxidation to $C^{14}O_2$ are shown in table 1. The labeled arachidonate was well absorbed by all groups, and no significant differences were noted between the groups. Of the amount absorbed 6 to 10% was oxidized to $C^{14}O_2$ in the 6 hours of the experiment. Although the difference in

[3] We are indebted to Dr. George Popják, London, for the generous use of his gas-liquid radiochromatograph for the analyses of the ethyl arachidonate-1-C^{14} and most of the samples described in this paper.

[4] The following quantities (in grams) were supplied by 100 g of diet: vitamin-test casein, 20; sucrose, 70; Hubbell, Mendel and Wakeman (5) salt mixture, 4; cellulose flour, 4; vitamin mixture in casein, 2. The latter supplied the following (in milligrams) per 100 g of diet: inositol, 100; thiamine·HCl, 5; riboflavin, 5; p-aminobenzoic acid, 5; niacin, 20; Ca pantothenate, 60; menadione bisulfite, 10; folic acid, 2; vitamin B_{12}, 0.0004; biotin, 0.0024; *d*-α-tocopheryl acetate, 3.4; and choline chloride, 100. An oil containing vitamins A and D provided 2400 USP units of vitamin A and 340 USP units of vitamin D/100 g of diet.

[5] Crisco, Procter and Gamble, Cincinnati, Ohio.

[6] Thixotropic gel powder, (trademark of Godfrey L. Cabot, Inc.), obtained from Packard Instrument Company, La Grange, Illinois.

TABLE 1

Intestinal absorption and oxidation to $C^{14}O_2$ of arachidonate-1-C^{14}

Experimental group	No. rats/ group	Intestinal absorption *	Expired C^{14} O_2 **
		% of administered dose	*% of absorbed dose*
Controls	7	69 ± 3 [1] (59–82) [2]	9.6 ± 1.0 (6.8–13.7)
Deficient	5	77 ± 11 (43–99)	6.6 ± 1.3 (4.7–11.2)
Deficient-supplemented	7	74 ± 6 (52–94)	10.1 ± 2.2 (4.3–20.6)

* Differences between means not statistically significant.
** Differences between means not statistically significant except for borderline significance between deficient and controls ($P = 0.075$).
[1] Mean ± SE.
[2] Numbers in parentheses indicate range.

mean values for deficient and control rats is only of borderline statistical significance ($P = 0.075$). The data in table 1 as well as inspection of the data of the individual animals indicate that the deficient animals oxidized less of the administered C^{14}-arachidonate to $C^{14}O_2$. The similarity of the mean values of controls and animals fed the supplement is marked.

Of the absorbed C^{14}-arachidonate only a minor amount was noted in the individual organs analyzed. The bulk of the activity was in the total carcass extract so that absorbed C^{14}-arachidonate was deposited generally in the body. The total amount of activity observed in long-chain fatty acids of the extracted tissues and carcass, plus the activity found in the $C^{14}O_2$ and in feces, did not exceed 75% of the total counts administered. The balance of the C^{14} activity appeared to be present in water-soluble compounds obtained after hydrolysis of the carcass. These could in large part be extracted from an acid solution by exhaustive extraction with diethyl ether, but further identification has not been made. Similar observations have previously been made in pyridoxine-deficient and normal animals given C^{14}-linoleic acid.[7]

The incorporation of the C^{14}-arachidonate into lipids of liver, heart, and gonads is shown in table 2. Of these 3 organs, the liver had the highest concentration (% of absorbed C^{14} per g tissue) as well as the greatest amount (% of absorbed C^{14} per entire organ). The livers of fat-deficient animals and those fed the supplements had lower amounts of C^{14} than the controls. The difference between mean values of controls and fat-deficient animals was statistically significant; that between controls and those fed the supplements was not. These statements also hold true for liver if the values are calculated as concentrations.[8] Although C^{14} content of gonads of fat-deficient animals and of those fed the supplements also was lower than that of controls, these differences were not statistically significant. In the heart statistically significant differences were found in the C^{14} activity of controls compared with fat-deficient or supplemented animals, but the statistical significance disappeared if the results were expressed as concentrations. (The average weight of the hearts of controls was 0.80 g compared with 0.60 g for the deficient and 0.52 g for the fat-deficient animals fed the supplements).

Subcellular fractions of a sucrose homogenate of liver had a similar distribution of C^{14} in the 3 groups. The percentage of the counts in each fraction was averaged from the results obtained in the 3 animals and was as follows: nuclei and debris, 16; mitochondria, 56; microsomes, 16; and soluble supernatant, 12.

The distribution of C^{14} in the various total fatty acids in each organ was determined by gas-liquid radiochromatography and is shown in table 3. Because of the small samples available and low radioactivity in each sample, only a limited amount of information could be obtained. The data, therefore, are presented as C^{14}

[7] Kirschman, J. C. 1960 A study of the effects of age, sex, and pyridoxine-deficiency on the metabolism of polyunsaturated fatty acids in the rat. Ph.D. thesis, Vanderbilt University.

[8] The weights of livers of fat-deficient and supplemented rats averaged 4.21 and 4.70 g, respectively; the average liver weight of the control animals was 5.87 g. The differences between the first 2 groups and the controls are statistically significant ($P < 0.001$). The liver total fatty acids of controls (average, 128 mg) were significantly greater ($P < 0.001$) than those of the deficient (84 mg) or supplemented (73 mg), but differences in concentration of fatty acids (mg/g liver) between groups were not significant (controls 22.1 ± 1.1, fat-deficient 20.3 ± 1.0, and supplemented 18.5 ± 1.5).

TABLE 2

Incorporation of orally administered C^{14}-arachidonate into total fatty acids of various organs

Group	No. rats/group	Total incorporation			Absorbed C^{14}		
		Liver	Gonads	Heart	Liver	Gonads	Heart
		% absorbed C^{14}			*% per g tissue*		
Control	6	9.9 ± 1.4 [1]	0.14 ± 0.035	0.18 ± 0.018	1.74 ± 0.03	0.059 ± 0.011	0.23 ± 0.023
Fat-deficient	5	6.4 ± 0.65	0.07 ± 0.02	0.10 ± 0.023	1.55 ± 0.04	0.051 ± 0.012	0.17 ± 0.054
Fat-deficient supplemented	6	7.1 ± 1.1	0.07 ± 0.03	0.10 ± 0.018	1.81 ± 0.09	0.047 ± 0.015	0.21 ± 0.046
P values (control, fat-deficient)		0.03	0.17	0.019	< 0.001	0.7	0.15
P values (control, supplemented)		0.12	0.17	0.017	0.075	0.55	0.7
P value (fat-deficient, fat-deficient supplemented)					0.4		

[1] Average of group ± SE

activity in certain regions of the chromatogram. The greatest proportion of the C^{14} activity of the chromatogram was usually in the arachidonate peak, especially in normal animals. In the fat-deficient animals, particularly in the heart and gonads, a decreased proportion of activity was in arachidonate. This in great measure was due to the increased amount in the shorter chain acids, notably myristic, palmitic and stearic. The amount found in areas representing chain lengths or unsaturation greater than arachidonate (probably 20 and 22-carbon fatty acids of 4, 5, and 6 double bonds), or both, was somewhat variable although large quantities were observed usually in fat-deficient animals (especially heart and gonads). Supplementation of the animals' diets with arachidonate increased the proportion of C^{14} as arachidonate in heart and gonads.

Fractionation of total lipids from liver tissue of one animal from each group by silicic acid column chromatography resulted in 3 main fractions: cholesterol esters, phospholipids, and a mixture of glycerides and free fatty acids. The latter were separated from the glycerides by alkaline extraction. C^{14} distribution in the various fractions was not significantly different in the 3 groups. The largest amount of C^{14} was in the phospholipid fraction (about 65 to 75%) and the smallest in the cholesterol ester fraction (about 1 to 2%). C^{14} content in triglycerides was 5 to 15% and free fatty acids, from about 10 to 30%. The relatively large amount of free fatty acid and of C^{14} in the free fatty acids in these samples indicates extensive breakdown of lipids in storage. Therefore, more detailed analyses were not made. Gas-liquid radiochromatography of methyl esters of fatty acids from each of the lipid fractions from each of the animals confirmed the observations reported for total fatty acid that the major amount of C^{14} was contained in arachidonic acid (60 to 90%). Comparison between groups was not feasible because of limited data and of considerable variability. A large portion of C^{14} activity (10 to 30%), however, was present in material of retention time greater than arachidonate and a smaller amount (5 to 10%) in compounds of retention time equivalent to that for stearic and palmitic.

TABLE 3

Distribution of C^{14} in total fatty acids of liver, heart, and testes

Fatty acids [1]	Liver [2]			Heart [3]			Testes [3]		
	Control	Fat-deficient	Supplemented	Control	Fat-deficient	Supplemented	Control	Fat-deficient	Supplemented
	% of total counts collected			*% of total counts collected*			*% of total counts collected*		
12:0 through 18:1	5.3 3.7	12.0 12.1	11.9 7.1	trace	18.5	8.8	trace	17.2	17.1
> 18:1 < 20:4	7.3 5.0	4.0 8.0	5.1 10.3	trace	4.6	5.9	trace	26.2	14.3
20:4	66.7 75.0	72.2 56.4	68.2 67.4	86.8	38.1	78.3	96.1	33.4	70.5
> 20:4	21.7 16.3	11.8 23.5	14.8 15.2	13.2	38.8	7.0	trace	23.2	trace

[1] Nomenclature of Farquhar et al. (12).
[2] Values for liver represent 2 rats from each group.
[3] Values for heart and gonads represent one pooled sample of several rats from each group.

The concentration of C^{14} in serum fatty acids was not significantly different in the 3 groups.

DISCUSSION

The small amount of C^{14} activity available for these studies restricted the amount of information which could be obtained and to some extent the nature of the observations which could be made. The data given in this paper are, therefore, presented with the realization that a time study of tissue activity would have made clearer the interpretation of isotopic data. Nevertheless, since at the end of 6 hours, absorption of C^{14} was still apparently taking place, it has been assumed that the conditions observed in the organs studied represent the metabolic state during the time of absorption and transport from intestines to body tissues and organs.

The organs selected for this study were those which accumulate relatively large amounts of 5, 8, 11-eicosatrienoic acid in essential fatty acid deficiency. That essential fatty acid deficiency was present in the experimental animals was proved by the concentration of 5, 8, 11-eicosatrienoic acid in the organs taken for analysis (5 to 12% of total fatty acid). Acute supplementation with arachidonic acid did not change significantly this amount although it increased the amount of arachidonic acid in livers of this group from about 9% to about 20% of total fatty acids. At the time of killing, the deficient animals weighed about 50 g less than their pair-fed controls.

Absorption of C^{14}-arachidonate given by stomach tube was satisfactory for the 6-hour interval. Compared with our results of 69 to 77% absorption in 6 hours, Coots [9] has observed in thoracic duct-cannulated rats that peak absorption of C^{14}-arachidonic acid incorporated randomly into triglycerides of soybean oil was at 12 hours after administration by stomach tube. We noted no apparent defect in absorption caused by fat-deficiency.

Very little of the absorbed C^{14} was oxidized to CO_2 in 6 hours. Of the 6 to 10% C^{14} obtained as CO_2 up to about 3 to 5% could have been due to the C^{14}-contaminants in the administered dose. This is unlikely and it is reasonable to assume that figures obtained represent the amount of C^{14} activity from arachidonic acid that had been oxidized to CO_2. The CO_2 collected by Coots [10] for 51 hours after C^{14} administration to rats contained 38.6% of the administered dose. That some C^{14}-arachidonate was utilized by oxidation during the time interval used in our experiments was also shown by incorporation of C^{14} into fatty acids of 16- and 18-carbon chain lengths, presumably by synthesis from acetate units. No C^{14}-fatty acid of 20-carbon chain length of less unsaturation than a tetraene was observed in the gas-liquid radiochromatograms with the exception of a trace amount of activity immediately prior to arachidonate which was

[9] Coots, R. H. 1963 The metabolism of 1-C^{14}-arachidonic and 1-C^{14}-linoleic acids in the rat. Federation Proc., 22: 303 (abstract).
[10] See footnote 9.

present also in the administered material. Therefore, there appeared to be no direct biohydrogenation which kept the fatty acid chain intact. Cleavage of the carboxyl carbon of the administered C^{14}-arachidonate, of course, would result in loss of the tracer carbon. Stein and Stein (7) had reported no measurable saturation of oleic and linoleic acid by rat adipose tissue in 60 days. However, Dhopeshwarkar and Mead (8) reported evidence for hydrogenation of methyl elaidate in guinea pigs. Action of intestinal flora was not ruled out in their studies. Moore and Sharman (9) observed that eleostearic acid was partially hydrogenated in the intestinal wall of the rat, giving an acid absorbing at 232 $m\mu$.

Results of liver studies showed a greater incorporation of the absorbed C^{14} arachidonate into phospholipid than into other lipid fractions. Similar observations have been reported for linoleic acid incorporation by Dittmer and Hanahan (10), and this is to be contrasted with oleic acid which these authors reported to be incorporated preferentially into neutral lipids. Since Coots [11] reported that thoracic duct lymph collected for 12 hours after feeding C^{14}-arachidonate contained 90% of the C^{14}-activity in the glyceride fractions and only 9.9% in the phospholipids, active conversion of glyceride-arachidonate to phospholipid-arachidonate must have taken place soon after the labeled lipid was taken up by liver tissue.

Because the livers of fat-deficient rats had lower concentrations of arachidonic acid than those of normal rats, it was expected that C^{14}-arachidonate would be retained to a greater extent in the livers of the animals than in livers of controls. However, the opposite was the case: livers of deficient animals had lower C^{14} activity than controls. The proportion of C^{14} due to arachidonate in these samples was about the same; therefore, livers of control animals had retained more of the absorbed arachidonate than livers of the deficient. The difference between the 2 animals was not due to oxidation to $C^{14}O_2$ since the deficient animals had significantly less C^{14} in the expired air than did control animals. The amount transported to other tissues or organs could have been different, but heart and gonads of deficient animals had less C^{14} (per total organs) than did the corresponding organs of control animals. It is possible that conversion to small, water-soluble C^{14}-containing compounds accounts for the difference.

It is well known that the concentration of arachidonic acid in phosphatides is rather large. Our experiments showed that absorbed arachidonic acid is not only preferentially incorporated into the phosphatide fraction (liver), but the C^{14} activity, for the main part, was observed to reside in mitochondria and in microsomes in which phosphatides are major lipid components.

Although we have not identified chemically the various labeled polyunsaturated fatty acids derived from C^{14}-arachidonate, it was apparent that a number of compounds of retention time higher than that of arachidonic acid were labeled. Two of these had retention times similar to those of 5- and 6-double bond fatty acids of 22-carbon chain lengths. Incorporation of C^{14}-arachidonate activity in some of these compounds lends confirmation to results reported by Mohrhauer and Holman (11). In long-term feeding studies these workers showed a conversion of arachidonate to a 22-carbon, pentaenoic acid identified as 4, 7, 10, 13, 16-docosapentaenoic acid.

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Mineralization of a Cereal Diet as It Affects Cariogenicity [1,2]

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ABSTRACT This investigation concerns the effect of certain mineral alterations of a raw whole wheat diet on its cariogenicity for white rats. The alterations were: replacement of dietary $CaHPO_4$ by equivalent Na_2HPO_4 with and without 1% NaCl supplementation, the withdrawal of the dietary supplementation of phosphorus, addition of NaCl to cariogenic diets with and without $CaHPO_4$ supplementation. Littermate Sprague-Dawley strain rats were fed at weaning each set of comparative diets and maintained with them for 60 days. The caries produced were the smooth-surface type. Replacing $CaHPO_4$ by Na_2HPO_4 decreased cariogenicity of the diet, and NaCl supplementation was also cariostatic in the presence of dietary $CaHPO_4$. Removal of $CaHPO_4$ from the diet did not alter its cariogenicity. Supplementation by NaCl was cariostatic in the 2 diets formulated with and without $CaHPO_4$. The sodium content of the persistently cariogenic diets was approximately one-fifth that recommended as needed for growth of weanling rats. It is suggested that inadequate sodium is related to the cariogenicity of the high cereal reference diets.

The consistent incidence of smooth-surface caries in white rats maintained with a high raw whole wheat, relatively low glucose diet, led to its use in studies of dietary factors which may alter cariogenicity. Inorganic and organic phosphates have been shown to be cariostatic when added to whole wheat diets (1–4). The addition of Na_2HPO_4 to a whole wheat diet has been shown to be cariostatic, but $CaHPO_4$ not to be. Both phosphates, however, were found to be cariostatic in a purified high sucrose diet (5). McClure (3) has indicated that NaCl in a whole wheat diet may be a factor in making insoluble dietary $CaHPO_4$ cariostatic.

This report deals with the effect of alterations in the mineralization of this whole wheat diet, particularly phosphate and sodium, as they affect its cariogenicity.

EXPERIMENTAL

The experimental rats were of the Sprague-Dawley strain, bred in this laboratory from a colony which originated from the National Institutes of Health. Rats, weighing 35 to 50 g, were fed each diet at weaning. Littermates, without respect to sex, were assigned to the diets within a series. The animals were housed 2/cage and received distilled water and food ad libitum. The food intakes were recorded twice a week, and the rats weighed weekly.

The base diet contained 78.5 to 79.0% raw whole wheat (ground to a flour); 18% glucose monohydrate;[3] 2.0% liver powder; calcium and phosphate additions varied with the trials. A vitamin A, D, and E supplement was given orally to each rat weekly.

Comparisons were made of the caries produced in rats fed the standard whole wheat diet, and the diet, supplemented by 1% NaCl, the diet altered by substitution of equivalent Na_2HPO_4 for $CaHPO_4$, the diet with Na_2HPO_4 and 1% NaCl supplementation (table 1, series A).

In an effort to restrict the role of phosphate to a systemic one (not oral), trials were made to establish a second reference diet with cariogenicity comparable to that of the one routinely in use. Such a diet was evolved by eliminating phosphate supplementation, but establishing a favorable Ca-to-P ratio by $CaCO_3$ supplementation to balance the phosphorus of the raw whole wheat and liver powder of the diet. The phosphorus of diet B10 was chiefly phytin phosphorus and thus not readily available in the oral cavity. There was the possibility that eliminating $CaHPO_4$ might in-

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[3] Cerelose 2001, Corn Products Company, Argo, Illinois.

TABLE 1

Effect of varying mineralization of raw whole wheat diets; supplementation of diets, protein, calcium, phosphorus and sodium analyses, caries experience, growth and food intake

Diet series	A100	A101	A102	A103	B100	B10	C100	C100p	D10	D11	D12
Diet variables											
Whole wheat, %	78.52	77.52	77.83	76.83	78.52	78.80	78.52	78.52	78.90	78.40	77.90
Glucose monohydrate, % [1]	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Liver Powder, %	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
$CaHPO_4$, %	0.88	0.88	—	—	0.88	—	0.88	0.88	—	—	—
$CaCO_3$, %	0.60	0.60	1.25	1.25	0.60	1.20	0.60	0.60	1.10	1.10	1.10
Na_2HPO_4, %	—	—	0.92	0.92	—	—	—	—	—	—	—
NaCl, %	—	1.00	—	1.00	—	—	—	0.60	—	0.50	1.00
Analyses											
Protein (N × 6.25), %	9.8	9.4	9.6	9.8	10.5	10.8	10.4	10.4	10.6	10.5	10.3
Calcium, %	0.56	0.56	0.56	0.56	0.54	0.53	0.67	0.67	0.52	0.53	0.53
Phosphorus, %	0.52	0.46	0.55	0.52	0.51	0.32	0.51	0.51	0.27	0.26	0.31
Sodium, %	0.009	(0.402) [2]	(0.307)	(0.700)	0.009	0.011	0.012	(0.248)	0.011	0.250	0.340
Carious experience											
No. rats	32	34	34	34	38	38	24	23	33	34	36
Carious rats, %	100	94	82	77	95	95	100	100	94	88	86
Avg no. carious teeth/rat	4.5 ± 0.32 [3]	3.1 ± 0.30	1.8 ± 0.22	1.5 ± 0.21	2.3 ± 0.17	2.0 ± 0.13	4.8 ± 0.33	3.8 ± 0.39	3.8 ± 0.33	1.9 ± 0.25	2.4 ± 0.29
Avg carious score/rat	10.4 ± 1.02	6.0 ± 0.78	3.2 ± 0.47	2.4 ± 0.33	4.3 ± 0.33	3.5 ± 0.30	12.0 ± 1.27	7.8 ± 1.21	7.6 ± 0.91	3.4 ± 0.60	4.1 ± 0.58
Avg wt increment, g/rat/day	1.2	1.2	1.1	1.1	0.9	1.0	1.0	1.5	0.9	1.7	1.8
Avg food intake, g/rat/day	8.7	8.4	8.0	8.0	8.8	8.9	8.9	9.2	8.5	9.9	10.2

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

[2] Numbers in parentheses indicate analyzed + calculated sodium in salts.

[3] SE.

crease cariogenicity. However, the 2 diets, B100 and B10, were shown to be comparably cariogenic (table 1).

The possibility that sodium supplementation, not as a phosphate, of these 2 reference diets, 100 and 10, was cariostatic was tested: a) by comparison of the caries produced in rats fed the raw whole wheat reference diet with 0.88% $CaHPO_4$ addition and this diet supplemented by 1% NaCl (table 1, A100 and A101); b) by comparison of the caries produced in rats fed reference diet 100, when one set of littermates received NaCl pellets, permitting voluntary supplementary NaCl intake (table 1, C100 and C100p) (the pellets were made by mixing 20 g NaCl to 80 g of the diet and adding water to make thorough mixing possible; the dough was divided uniformly and rolled into balls and baked 5 to 7 hours at 93°; the pellets weighed 8 ± 0.3 g; they were hard and durable and were always available in the cages); and c) by comparison of the caries produced in rats fed the raw whole wheat reference diet without phosphate addition and this diet supplemented by 0.5 and 1.0% NaCl (table 1, series D).

All supplementation was made at the expense of the raw whole wheat. The diets were analyzed for nitrogen, ash, calcium, and phosphorus. The nitrogen was determined by the Kjeldahl method, calcium by the method of the AOAC (6), and phosphorus by the method of Simmons and Robertson (7). In certain trials diets were analyzed for sodium.[4]

Inoculation of the diets with fecal matter was routinely carried out in diet series C and D. This practice was adopted to provide a degree of uniformity of bacterial contamination (8).

After 60 days on experiment, the rats were killed and the heads autoclaved to facilitate removal of the jaws. The lower molar teeth were examined for smooth surface caries as described by McClure (1). Fissure caries were not observed. Comparisons of cariogenicity are possible only within each series.

RESULTS

The result of substitution of Na_2HPO_4 for $CaHPO_4$ in the raw whole wheat, series A, was a significant decrease ($P < 0.01$) in the carious score per rat (table 2).

The data show that 1% NaCl supplementation to the raw whole wheat diet was also significantly cariostatic ($P < 0.01$, carious score per rat). The diets A102 and A103, containing Na_2HPO_4, were significantly more cariostatic than their counterparts containing $CaHPO_4$, diets A100 and A101, ($P < 0.01$, carious score per rat), but NaCl supplementation did not enhance the cariostatic action provided by Na_2HPO_4 alone. These results raised the question of the adequacy of sodium in this reference whole wheat diet and a possible relationship of a low sodium content to its persistent cariogenicity.

In the development of a second cariogenic reference diet in which the available phosphate could be considered chiefly systemic, the diet which best fulfilled the requirements had no added phosphate but $CaCO_3$ was added (table 1, diet B10). The caries experience of the rats fed the 2 diets, B100 and B10, was similar. There was no significant difference in the carious score per rat (table 2).

[4] Rosner-Hixon Laboratories, 7737 S. Chicago Avenue, Chicago.

TABLE 2

Significance of differences (t test) between mean total carious score per rat fed diets A, B, C and D [1]

Diet series	A100	A101	A102	B100	C100	D10	D11
A101	3.44						
A102	6.38	3.04					
A103	7.38	4.09	1.41				
B10				1.82			
C100p					2.39		
D11						3.85	
D12						3.24	0.84

[1] *t* value needed for significance: 1%, 2.66; 5%, 2.00.

The trials in which sodium supplementation, not as a phosphate, but as NaCl, in both reference diet 100, in which the action with phosphate might be oral, and in reference diet 10 in which the action could be assumed to be systemic are presented in tables 1 and 2, diets A100—A101, and series C and D.

When NaCl was provided as pellets, diet C100p, and the intake was voluntary, the sodium chloride was calculated to have been 0.6 ± 0.06 of the diet or equivalent to 22 mg sodium/rat/day. This voluntary intake was cariostatic for these rats as compared with littermates fed the same diet without access to salt, diet C100. Since dietary $CaHPO_4$ was available in these 2 trials, the cariostatic action may have been in the oral cavity.

The comparison of the cariogenicity of reference diet 10 and of the diet containing 0.5 and 1.0% NaCl are presented in table 1, diet series D. At both levels NaCl was cariostatic ($P < 0.01$, carious score per rat) and to a comparable degree (table 2). This cariostatic action was considered to be systemic.

These data showed improved growth as well as reduced caries experience when NaCl was included in the diets. This improved growth was noted in all trials in this laboratory when NaCl was present in these raw whole wheat diets, except the initial trial (table 1, series A). These observations led to the analysis of sodium in the diets. Both reference diets, 100 and 10, had a sodium content of 11 to 12 mg/100 g of diet, and hence provided an intake of approximately 1 mg/rat/day compared with the 5 mg suggested as needed for growth of weanling rats (9).

When sodium intake was 0.250% of the diet or approximately 25 mg/rat/day, diet D11, and when intake was voluntary and 23 mg/rat/day, diet C100p, these levels were cariostatic. In trial D11 the cariostatic effect could not be attributed to a solubilizing effect of NaCl on dietary phosphate, although it might have been in trial C100p.

DISCUSSION

The auxiliary role of sodium appears to have been involved in many of the studies on the cariostatic action of phosphates. The phosphates tested have often been sodium salts.

Van Reen and Ostrom (5) have shown that in a high sucrose purified diet both Na_2HPO_4 and $CaHPO_4$ were cariostatic, but that only Na_2HPO_4 was so in a whole wheat diet. However, this purified diet contained a 2% HMW salt mixture (10) and 30% casein. Casein contains about 10 times the sodium that whole wheat does and 2% HMW mixture provides 55 mg/100 g diet or approximately 5 mg/rat/day.

McClure (3) demonstrated the cariostatic effect of including NaCl in a wheat cereal diet in which the phosphate was $CaHPO_4$. He suggested it was likely that $CaHPO_4$ was solubilized by NaCl resulting in an increased availability of calcium and phosphate ions within the oral cavity; that this could be the cause of the cariostatic effect of $CaHPO_4$ when added to bread which contained NaCl; and that Na_2HPO_4 may be cariostatic within the oral cavity.

The observation of suboptimal levels of sodium in the whole wheat reference diets and the cariostatic effect of adding sodium, not a phosphate, does not remove the possibility of an auxiliary role of sodium in the utilization of phosphate for decreasing caries. It does, however, suggest a systemic not an oral action.

It is not suggested as a result of this reported work that sodium ion is cariostatic, but that because of the suboptimal sodium level of this whole wheat diet there is a metabolic impairment which may be responsible for its cariogenicity.

Such an impairment may be that demonstrated in 1937 by Kahlenberg and his associates (11), who published a study on the effect of sodium deficiency on utilization of energy-producing nutrients and protein. The inadequate diet was predominately maize (78.9%) and contained 0.007% sodium. It was supplemented by Na_2CO_3 to furnish an adequate sodium level, 0.502% in the second diet. Rats were pair-fed these diets for 70 days following weaning. The food intake was the same and shown to be equally digestible for the 2 groups. For the supplemented group, weight gain was 63.3 g, nitrogen retention 2.46 g or 18.5% of the food nitrogen; for the deficient group the correspond-

ing values were 31.5 g, 1.40 g, and 10.5%. The authors state that "sodium deficiency unfavorably affected the appetite, the increase in weight, the storage of energy and the synthesis of fat and protein."

Because of the broad and fundamental functions of sodium in the body, it is likely unsafe to define a specific role for it as it affects caries production; however, it is suggested that the suboptimal level of sodium in these high whole wheat diets formulated without the addition of sodium salts, adversely affected the utilization of an initially poor protein and the synthesis of protein by the growing rat, and that the cariogenicity of the diet is related to an augmented protein inadequacy rather than to a sodium deficiency directly. This interpretation would rationalize a variety of reported observations.

McClure (12) demonstrated a high millet diet to be cariogenic and lysine supplementation not to relieve the cariogenicity of the diet nor to promote growth. However, he was able to show the millet to be lysine-deficient by a growth test with an even higher millet diet supplemented by 1% lysine. The diet used in the growth study contained 2% HMW salt mixture (10) and 4% cottonseed oil. The rats fed this diet developed no caries and the author suggested "perhaps two factors present in this nutritionally adequate diet, i.e., the 4.0% cottonseed oil or the HMW salt mix may have prevented the development of dental caries."

In our own laboratory dried egg white and casein supplementation of the cariogenic diet 100 was shown to decrease caries and increase growth more than an equivalent gluten supplementation (8). Lysine supplementation of this same diet was not cariostatic nor did it promote growth. The sodium content of these diets has now been calculated from the analyzed sodium values of the component parts. The only contribution of sodium to the diet was by casein and egg white. These contributions were small, but they doubled or tripled the content, although the level in the diet was still below that suggested as needed for growth. The gluten had no more sodium than the wheat; hence the limited cariostatic action it displayed must have been dependent on the heightened amount of protein in the diet.

It appears that the cariogenic properties of high cereal diets we have reported (13) may be related to the low sodium content of the cereals and the diets as formulated rather than a specific property of the cereals.

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The Cholesterol-lowering Effect of Commercial Diet Fed to Germfree and Conventional Rats [1]

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ABSTRACT Germfree and conventional rats were fed laboratory-made semi-purified diets, a commercial diet fortified for heat sterilization, and semi-purified diets incorporating the lipid fraction isolated from the commercial diet. In both germfree and conventional animals total serum and liver cholesterol values in animals fed the commercial diet were, on the average, 20 to 30% lower than the levels for the animals maintained with the semi-purified formulas. Upon changing from the semi-purified to the commercial diet, serum cholesterol values in both animal groups decreased by approximately the same amount. These changes were completely reversible. When the lipid fraction extracted from the commercial diet was used to replace the corn oil in the semi-purified formula, similar results were obtained. In all experiments where germfree and conventional animals were compared, the data showed a striking similarity. The results suggest that the commercial diet contains a cholesterol-lowering lipid fraction which acts directly on cholesterol metabolism and not via an effect on the intestinal microflora.

Although dietary components greatly influence cholesterol metabolism, in many cases the question arises whether this occurs via an effect on the intestinal microflora or by some direct systemic influence on the animal (1). The germfree animal is the ideal tool for the study of the effect of dietary components on the systemic cholesterol metabolism because of the obvious absence of microbial influences.

The influence of the intestinal microflora on cholesterol and bile acid metabolism has been demonstrated by the effect of the addition of antibiotics to the diet (2–4) and by experiments with germfree animals (5, 6). Harper and Elvehjem (7) suggested that dietary fat, protein and carbohydrate all have a direct or indirect effect on the intestinal microflora. Portman et al. (8) reported that feeding commercial chow to rats resulted in a higher excretion of fecal bile acids than feeding purified diets containing starch or sucrose, and speculated that changes in the intestinal flora due to the feeding of different carbohydrates may affect the bile acid cycle.

In results reported from our laboratory (6) conventional rats fed commercial chow consistently showed substantially lower serum and liver cholesterol levels than rats maintained with a variety of well balanced semi-purified diets. To confirm the cholesterol-lowering action of the commercial diet and to gauge the importance of the microbial factor in this phenomenon, experiments were designed to test similar diets with germfree animals. In this paper we report the results of feeding a commercial diet (fortified for nutritional adequacy after sterilization) and the lipid fraction isolated from that diet, to both germfree and conventional rats. The results indicate a direct systemic effect of the diet, and also of the dietary fat, on cholesterol metabolism, leading to lower cholesterol concentration in both serum and liver.

MATERIALS AND METHODS

Animals. Lobund-strain male rats of Wistar origin were used in all experiments. The germfree rats were taken from the germfree colony housed in the Reynier system and transferred to Trexler plastic isolators for the duration of the experimental period. The conventional rats were taken from a colony which is kept closely related to the germfree by introduc-

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ing ex-germfree male breeders with every production cycle. These rats were not kept in isolators, but otherwise housing conditions of germfree and conventional animals were comparable.

Diets. Diet L-356 is the major general purpose, steam-sterilizable semi-purified diet used at the Lobund Laboratory (9), and much of the earlier information was obtained with this diet. The diets of the L-474 series were more simplified formulas in which liver powder and yeast extract had been omitted, but which were otherwise closely related to L-356 (table 1). Diet L-474E1 contained vitamins in concentrations which met only the normally accepted requirements, meaning that this diet was not fortified to undergo sterilization. Diet L-474E5 contained vitamins similar to those in diet L-356, except that ascorbic acid was omitted. All these diets contained less than 25 mg cholesterol/100 g diet.

Diet L-474E6 was essentially the same as L-474E5 except that extracted "oil R" was used to replace all corn oil. A similar replacement in diet L-356 yielded formula L-356E1. "Oil R" was obtained by grinding commercial rat pellets [3] to a powder and extracting the lipids with *n*-hexane at 62° for 24 hours. Since 95% of the vitamin A originally present in the pellets could be recovered from the extracted lipids, it was assumed that practically all fat in the diet was extracted into the hexane. About 4% of the diet weight was obtained as lipid after a thorough evaporation of hexane. The iodine number was 110. The corn oil used to prepare diets L-356, L-474E1 and L-474E5 showed an iodine number of 110 to 115.

Commercial rat pellets were ground and then fortified for steam sterilization with L-lysine, 0.6 g, DL-methionine, 0.3 g, *i*-inositol, 50 mg, thiamine·2 HCl, 5.8 mg, and vitamin A, 190 IU in 1 g of corn oil, per 100 g of original diet to give formula RF. All diets were steam sterilized unless indicated otherwise.

Tissue samples. In experiments in which total serum cholesterol values were determined following one or more diet changes, 1 ml of blood was obtained by heart puncture from all animals before the change. Thereafter usually one-half of the animals were bled at semi-weekly intervals during the experimental period, resulting in each animal being subjected to heart puncture once a week. Only terminal liver samples were obtained.

Determination of total cholesterol. Blood taken by heart puncture was assayed

[3] Rockland Rat Pellets, A. E. Staley Manufacturing Company, Decatur, Illinois.

TABLE 1

Composition of diet L-356 and related formulas

	L-356	L-474E1	L-474E5
	g/100 g	*g/100 g*	*g/100 g*
Casein [1]	20	20	24
DL-Methionine	—	0.3	0.3
Rice flour	58.5	—	58.5
Rice starch	—	58.5	—
Liver powder	2	—	—
Yeast extract	2	—	—
Corn oil	5	3	3
Powdered cellulose [2]	5	10	5
Salts L-II [3]	5	5	5
Ascorbic acid	0.2	—	—
i-Inositol	0.1	0.1	0.1
Choline-biotin additive [4]	—	1	—
B-vitamin mixture 75 [3]	0.5	0.1	0.5
Ladek 3 in corn oil [5]	2	—	2
Ladek 52 in corn oil [6]	—	2	—

[1] Labco, The Borden Company, New York.
[2] Cellophane Spangles, Microfibre, Inc., Pawtucket, Rhode Island.
[3] Zimmerman and Wostmann (10).
[4] Choline chloride: 166 mg; biotin: 0.02 mg; rice starch carrier: 834 mg.
[5] Ladek is a term coined by this laboratory to designate fat-soluble vitamin mixtures. Ladek 3: vitamin A, 800 IU; vitamin D_3, 100 IU; mixed tocopherols, 150 mg; menadione, 10 mg (10).
[6] Ladek 52: vitamin A, 800 IU; vitamin D_3, 100 IU; mixed tocopherols, 7.5 mg; menadione, 1 mg.

by a method based on the Tschaugaeff reaction as outlined by Hanel and Dam (11); otherwise all serum and liver cholesterol was determined with a modification of the Lieberman-Burchard method (6). Based on an internal standard of free cholesterol, the results of both methods for total serum cholesterol were in agreement within the limits of the possible experimental error.

Experimental series. In a preliminary experiment the effect of diet L-474E1 on total serum cholesterol was tested. This diet was not fortified for sterilization and was fed in nonsterile form to conventional rats (table 2). In the first series designed to test the influence of the commercial diet on serum cholesterol, 3-month-old male rats, both germfree and conventional, were changed from diet L-356 with which they had been reared, to the fortified commercial formula RF, and then changed back to diet L-356 (fig. 1). The germfree group was then subjected to a second change to formula L-474E6, containing the lipid fraction "oil R" from the commercial diet. The second part of this experiment was paralleled by a series in which conventional rats reared with formula L-474E5 were changed to diet L-474E6. Thereafter both groups were changed back to formula L-474E5. Experimental periods with formula RF or with diets containing "oil R" lasted from 3 to 5 weeks.

In the second and third series, the effects of the commercial diet on both serum and liver cholesterol concentrations were determined. In the second experiment germfree and conventional rats reared for 3 months with diet L-356 were subsequently changed to the fortified commercial formula RF (table 3). In this series blood samples were obtained before the diet change, but final liver cholesterol levels had to be compared with known average values of animals fed diet L-356. The third series comprised only germfree rats reared with diet L-356 (table 4). At the age of 3 months one-third of these rats continued to receive diet L-356, one-third were changed to formula RF and one-third were changed to L-356E1, a formula in which all corn oil had been replaced with the lipid fraction extracted from the commercial rat pellets.

RESULTS AND DISCUSSION

Cholesterol levels in conventional rats fed the various sterilized experimental diets used in the work with germfree animals were substantially higher than the values found using commercial-type diets (table 2). At one time it was thought that the fortification of these diets with extra protein and vitamins, required because of the necessary heat sterilization (9), could create a nutritional imbalance which would lead to abnormal cholesterol levels. However, even when the levels of the various nutrients were brought within the limits of the generally accepted normal requirements as in diet L-474E1 (table 1), and the diet was fed in nonsterilized form, the serum cholesterol levels remained high relative to the values noted upon the feeding of commercial-type diets (table 2). It was then postulated that serum cholesterol concentrations of about 120 mg/100 ml were "normal" for the strain of rats used at the Lobund Laboratory and that the lower values determined for commercial-type diets actually represented a depression of cholesterol levels. Since all diets mentioned contain very little cholesterol,

TABLE 2

Total serum cholesterol values in 3- to 4-month-old male conventional rats fed various complete diets [1]

Diet	Samples	Body wt	Cholesterol
		g	*mg/100 ml*
L-356 sterile	> 20	359	122
L-474E1 nonsterile	12	329	114
L-474E5 sterile	> 20	368	116
L-462 [2] sterile	14	405	130
RF	> 20	330	84

[1] Data compiled from a number of different experiments.
[2] Zimmerman and Wostmann (10).

TABLE 3

Effect of fortified commercial diet RF on total serum and liver cholesterol in germfree (series 12 A) and conventional (series 12 B) male rats previously fed semi-purified diet L-356 [1]

	Germfree				Conventional			
	21–100 days		100–120 days		21–100 days		100–120 days	
	Diet L-356		Diet RF		Diet L-356		Diet RF	
	Series PA [2]	Series 12A	Series 12A		Series PB [2]	Series 12B	Series 12B	
Body wt, g	334 ± 7 [3]	334 ± 6	326	± 2	359	± 11	349 ± 11	358 ± 10
Liver wt, %	3.34 ± 0.10	—	2.72	± 0.07	3.43	± 0.09	—	3.81 ± 0.07
Serum cholesterol, mg/100 ml	106 ± 4	104 [4] ± 3	85 [5]	± 2	127	± 5	125 [4] ± 3	90 [5] ± 5
Liver cholesterol, mg/100 g	403 ± 18	—	310 [6]	± 8	326	± 7	—	259 [6] ± 13
Liver cholesterol, total, mg	40.7 ± 2.0	—	27.6 [6]	± 1.4	40.7	± 2.1	—	35.9 [6] ± 1.7

[1] Averages on 10 or more animals in each experimental group.
[2] Comparable data taken from B. S. Wostmann and N. L. Wiech, Am. J. Physiol., *201:* 1027, 1961.
[3] SDM.
[4] Blood obtained by heart puncture before change of diet.
[5] Difference from control value (L-356) significant at $P \leqq 0.01$.
[6] This SDM value indicates a significant difference from values obtained in an earlier series (PA and PB).

the question then arose whether this depression was caused by some agent in the diet affecting the reabsorption, biosynthesis, distribution or catabolism, or whether some difference in the intestinal microflora had caused a shift in cholesterol metabolism in the rats fed the commercial diet. Portman et al. (8) had observed that more bile acid was excreted in the feces of rats fed commercial diet. This appeared to fit well with our observation, and had been explained by Bergstrom (12) as indicating an effect via the intestinal microflora. The data in figure 1 indicate, however, that upon change from a semi-purified to a commercial-type formula, both germfree and conventional rats demonstrated a sharp decrease in total serum cholesterol. The effect of the diet change became obvious within a few days, and was totally reversible. In a similar experiment (table 3) it was demonstrated that a change to the commercial diet lowers cholesterol concentrations in serum and liver alike, and again independently of the presence of an intestinal microflora.

Under the assumption that some factor in the diet, possibly a lipid fraction, was responsible for the lowering of the cholesterol levels, the commercial rat pellets were extracted with *n*-hexane and the lipid fraction thus isolated used to replace the corn oil in diets L-474E5 and L-356. Figure 1 illustrates the serum cholesterol-lowering effect of this fraction, which again was reversible and independent of the presence of an intestinal microflora. The data in table 4 demonstrate that, in the germfree rat, the replacement of corn oil by this lipid fraction led to lower cholesterol levels in both the serum and the liver and that this effect was quite comparable with the result produced by a change to the (fortified) RF diet.

Collectively these results indicate that a lipid fraction in the commercial diet drastically influences systemic cholesterol metabolism. Gas-liquid chromatography has shown the extracted lipid fraction to be a very complex mixture. The iodine number of 110 was of the same order as that of the corn oil used in the other diets. Preliminary chromatographic separation on Fluorisil [4] columns did not produce any

[4] Floridin Company, Hancock, West Virginia.

TABLE 4

Effect of fortified commercial diet RF and of its lipid fraction "oil R" on total serum and liver cholesterol in germfree rats previously fed semi-purified diet L-356

No. animals	Diet L-356 (continued)	Diet L-356E1 (with "oil R")	Diet RF
	6	8	6
Body wt, g	368 ± 8 [1]	367 ± 11	304 [2] ± 14
Liver weight, %	3.27 ± 0.08	3.57 ± 0.13	2.99 ± 0.13
Serum cholesterol, mg/100 ml	113 ± 6	94 [3] ± 2	92 [2] ± 5
Liver cholesterol, mg/100 g	364 ± 10	281 [2] ± 12	260 [2] ± 10
Liver cholesterol, total, mg	43.9 ± 2.2	36.6 [2] ± 1.5	24.7 [2] ± 1.3

[1] SDM.
[2] Difference from control value (L-356) significant at $P \leq 0.01$.
[3] Difference from control value (L-356) significant at P 0.01–0.05.

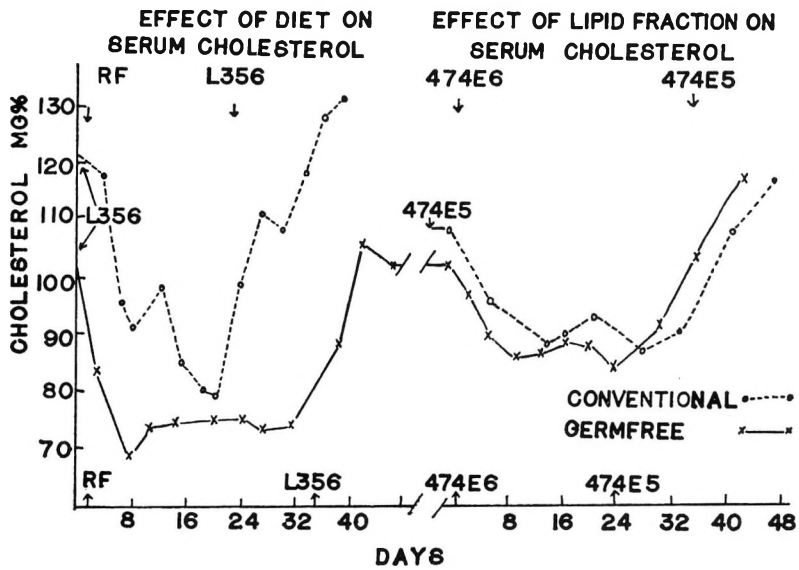

Fig. 1 Effect of dietary changes on serum cholesterol levels. Original diets indicated in figure. Diet changes indicated at top (conventional series) and at bottom (germfree series). L-356 and L-474E5: semi-purified diets; RF: fortified commercial diet; L-474E6: semi-purified diet containing the lipid fraction extracted from the commercial diet.

subfractions of much higher unsaturation. Obviously much more work will be needed before the actual isolation and identification of the active principle can be achieved.

The change from diet L-356 to formula RF was always accompanied with some loss in body weight. The data in table 4 show that this was not the cause underlying the decrease in cholesterol levels. In the experiments in which the only change in the diet was the replacement of corn oil with "oil R," body weights were not affected, but the decrease in cholesterol concentrations was of the same order.

Part of this loss in body weight can be ascribed to the weight loss which usually accompanies any change in diet. It has been our experience, however, that in conventional rats the gain in body weight with the various semi-purified diets used in the Lobund Laboratory was slightly higher than that with the commercial rat pellets. In recent work with germfree animals this has been confirmed. Male rats in the linear part of their growth curve showed a weight gain of between 30 and 40 g/week with the sterilized semi-purified formulas, whereas the gain with sterilized RF diet

was slightly under 30 g/week. This is presumably related to the lower fat and higher fiber content of the commercial formula, which may also necessitate a longer adjustment period in which weight loss can occur. Concurrent with a lower gain in body weight of the conventional rat during its growth period and with lower cholesterol levels during its lifetime, the conventional rat fed the commercial pellets shows a much longer life span (50%, survival time 836 days) than those fed semi-purified formulas (L-356: 654 days; L-462: 639 days).[5]

The reduction in cholesterol levels obtained by feeding the RF diet, or a semi-purified formula incorporating "oil R," to germfree rats unquestionably indicates an effect on systemic cholesterol metabolism. The similarity of the results in germfree and in conventional animals suggests, however, that also in conventional rats the cholesterol-lowering effect of this diet is mainly a direct one, not caused via influences of dietary components on the intestinal microflora. It is generally considered difficult to influence serum and liver cholesterol by dietary means in rats fed "normal" total amounts of carbohydrates, proteins and fats (13, 14). Wilkens and co-workers have based the explanation of the cholesterol-lowering effect of certain unsaturated fats in their experiments on the lower solubility of cholesterol in hypocholesterolemic oils (15), an explanation which appears independent of the presence or absence of an intestinal microflora. The above results portray the feasibility of a substantial reduction of body cholesterol pools via dietary means, while indicating that such a reduction can be independent, in the first approach, of changes induced in the intestinal microflora.

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Kidney Beans (*Phaseolus vulgaris*) and the Effectiveness of Vitamin E for Prevention of Nutritional Muscular Dystrophy in the Chick [1]

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ABSTRACT Groups of one-day-old chicks fed a vitamin E-free diet contracted nutritional muscular dystrophy (NMD) at the rate of 95 to 100%. The addition of 20 to 25 IU of vitamin E/kg of diet decreased the incidence to 5 or 6%. Adding raw kidney beans in addition to the vitamin E increased the incidence of NMD to 45 to 100%, indicating the beans contain an anti-vitamin E factor (PAT, Phaseolus antagonist to tocopherol). Extracting and autoclaving the beans indicated 2 antagonists to vitamin E; one, alcohol-soluble and heat stable and the second, not alcohol-soluble and heat labile. Evidence indicated that the alcohol-soluble antagonist was due to the unsaturated fats present. The specific nature of the second antagonist is as yet unknown.

Hogue et al. (1) reported that a ration containing raw kidney beans when fed to ewes was effective in the production of nutritional muscular dystrophy (NMD) in their lambs. Autoclaving the beans decreased the incidence of NMD and the investigators proposed the presence of a heat-labile vitamin E antagonist in kidney beans. However, they also reported that autoclaved beans exhibited negative vitamin E activity in the prevention of exudative diathesis in the chick.

Other legumes have been reported to have anti-vitamin E activity. Sanyal (5) reported peas, *Pisum sativium*, contained a factor which decreased the effectiveness of vitamin E in the prevention of fetal resorption in the rat. Pudelkiewicz and Matterson (4) reported that alfalfa contained a lipid-soluble material which decreased the biological value of vitamin E for chicks by increasing vitamin E excretion.

Raw kidney beans are toxic to rats (3) but chicks are more resistant to the toxicity. In preliminary studies, diets containing 50% raw beans produced 100% mortality in young rats; however, chicks were able to survive and make slight gains when fed similar diets. Wagh et al. (7) also reported that chicks could survive with a diet containing 50% raw beans. Honavar et al. (2) isolated a hemagglutinin from kidney beans which caused a high mortality in rats when fed at levels as low as 0.5% of the diet. Twice this level of hemagglutinin produced no evidence of mortality in chicks (7).

The present studies were conducted to determine whether kidney beans affected the vitamin E requirement for the prevention of NMD in chicks.

EXPERIMENTAL

One-day-old, White Plymouth Rock male chicks from a commercial hatchery [3] were used in all experiments. The chicks were housed as groups in electrically heated battery brooders with wire-mesh floors. Lighting was maintained 15 hours per day. The negative control diet was similar to that used by Scott and Calvert (6) to develop NMD in chicks. The composition of this diet is shown in table 1. All diets were fed ad libitum. The appearance of characteristic white striations in the breast muscle, visible either through the skin or upon autopsy at the termination of the experiment, was considered positive for NMD. Three individual experiments were conducted as follows.

Experiment 1. Sixteen chicks, 4 days of age, were assigned at random to each of the dietary treatments listed in table 2.

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[3] Cobb's Pedigreed Chicks, Inc., Concord, Massachusetts.

TABLE 1

Composition of negative control diet

	%
Glucose [1]	60.88
Torula yeast [2]	10.00
Casein	5.00
Isolated soybean protein [3]	5.00
Stripped lard [4]	4.00
Cellulose [5]	3.00
Mineral mixture [6]	6.04
Vitamin mixture [7]	0.72
L-Arginine	1.00
L-Glutamic acid	1.50
Glycine	2.50
L-Leucine	0.20
DL-Methionine	0.10
DL-Tryptophan	0.06
Ethoxyquin [8]	0.0125

[1] Cerelose, Corn Products Company, New York.
[2] Kindly supplied by K. L. Cartwright, Lake States Sales Company, New York.
[3] ADM C-1 Assay Protein, Archer Daniels-Midland Company, Minneapolis.
[4] Distillation Products Industries, Rochester, New York.
[5] Solka Floc, Brown Company, Berlin, New Hampshire.
[6] Mineral mixture: (% of diet) $CaHPO_4 \cdot 2H_2O$, 2.71; $CaCO_3$, 1.492; KH_2PO_4, 0.867; NaCl, 0.600; $MnCl_2 \cdot 4H_2O$, 0.0463; $FePO_4 \cdot 4H_2O$, 0.265; MgO, 0.068; KI, 0.00026; Cu $(C_2O_4)_2$, 0.00119; ZnO, 0.006; $CoCl_2 \cdot 6H_2O$, 0.00017; $KHCO_3$, 0.21; $NaMoO_4 \cdot 2H_2O$, 0.00083.
[7] The vitamin mixture supplied the following per kg of diet: (in mg) thiamine, 10; riboflavin, 10; pyridoxine·HCl, 4.5; niacin, 50; inositol, 250; folic acid, 4; Ca pantothenate, 20; biotin, 0.20; menadione, 0.50; choline chloride (70%), 2200; and vitamin B_{12}, 20 μg; vitamin A, 5600 IU; vitamin D_3, 4410 ICU.
[8] Santoquin, Monsanto Chemical Company, St. Louis, Missouri.

The beans, when added to the basal diet replaced 5% Torula yeast, 2.5% casein, 2.5% soy protein and 40% glucose. When the diets contained 75% beans, beans replaced 3% cellulose, 3% casein, 3% soybean protein and 6% Torula yeast and all the glucose in the basal diet.

The beans were alcohol-extracted by mixing equal weights of beans and 95% ethyl alcohol in a Waring Blendor for 3 minutes, and then filtering. The extraction procedure was repeated 3 times. The alcohol was removed from the extract by evaporation at room temperature. All birds were continued on trial up to 28 days.

Experiment 2. Twenty chicks were randomly assigned to each of the treatments listed in table 3. A 7-day preliminary period in which all chicks received the basal diet preceded a 24-day experimental period. The alcohol extract was obtained in the same manner as in experiment 1. The acetone precipitates were obtained by adding acetone directly to the alcohol and extract. A white precipitate was obtained by adding acetone to the alcohol extract. The precipitate at room temperature (25°) was removed by centrifugation and another precipitate was obtained when the supernatant fraction was placed in −30°

TABLE 2

Effect of vitamin E and various kidney bean fractions on the incidence of nutritional muscular dystrophy, daily gain, diet intake and pancreas weight in the chick (trial 1)

Diet	Incidence of nutritional muscular dystrophy		Avg daily gain /chick [2]	Total diet intake /group	Avg pancreas wt
	No. [1]				
		%	*g*	*kg*	*g/100 g body wt*
1 Negative control	16/16	100	8.0	—	0.24
2 Basal (diet 1 + 20 IU/kg vitamin E) [3]	1/16	6	12.3	13.5	0.26
3 Basal + 50% raw beans	12/14	86	4.4	7.8	0.63
4 Basal + 75% raw beans	6/8	75	1.5	3.5	0.67
5 Basal + 50% autoclaved [4] beans	8/16	50	11.1	13.8	0.39
6 Basal + 75% autoclaved [4] beans	14/16	87	7.2	11.2	0.38
7 Basal + 50% alcohol-extracted beans	11/16	69	6.1	9.5	0.56
8 Basal + alcohol extract [5]	9/16	56	9.5	8.6	0.24
9 Negative control + 0.3% cystine	0/16	0	15.0	12.4	0.24
10 Negative control + 0.3% cystine + 50% raw beans	0/17	0	4.9	7.3	0.59
11 Chick starter [6]	0/16	0	20.6	—	0.24

[1] Indicates number affected over total number of normal plus affected.
[2] For chicks surviving to the end of the experiment only.
[3] Rovimix E, DL-α-tocopheryl acetate, Hoffmann-La Roche, Inc., Nutley, New Jersey.
[4] At a temperature of 118° for 30 minutes.
[5] Added to simulate a 200% bean diet.
[6] Chick Grow, Agway, Inc., Ithaca, New York.

cooler for 12 hours. This precipitate was removed and the solvents in the supernatant were evaporated and the residue retained. Thus, the alcohol extract was fractionated into 3 parts: 1) acetone precipitate at 25°; 2) acetone precipitate at −30°; and 3) residue. In this experiment the additions to the control diet replaced glucose only.

Experiment 3. Ten chicks were assigned at random to each of the treatments listed in table 4. For the NaOH extraction, 2 kg of finely ground raw beans were mixed with 12 liters of 0.2 N NaOH. After 24 hours the mixture was filtered through cheesecloth and the residue washed with water and dried with fans at room temperature. Ethyl alcohol (95%) was added to the filtrate until no more precipitate (protein) was formed and the mixture was filtered through cheesecloth. The residue was dried in a freeze-drier. All chicks were fed the basal diet for 14 days and the experimental diet for 14 days. The bean additions were made at the expense of glucose.

RESULTS AND DISCUSSION

Experiment 1. All the chicks fed the negative control diet developed NMD (table 2) (diet 1) but 20 IU of vitamin E/kg of diet (*dl*-α-tocopheryl acetate) (diet

TABLE 3

Effect of various kidney bean fractions and autoclaved kidney beans and linoleic acid on the vitamin E requirement of the chick (trial 2)

Diet	Incidence of nutritional muscular dystrophy		Avg daily gain [2]	Total diet intake
	No. [1]			
		%	*g*	*kg*
1 Negative control	19/20	95	11.9	12.6
2 Basal (diet 1 + 20 IU of vitamin E/kg) [3]	1/20	5	12.0	12.6
3 Basal + 50% raw beans	9/20	45	9.6	13.0
4 Basal + 50% autoclaved [4] beans	11/20	55	14.5	15.0
5 Basal + 50% autoclaved [5] beans	4/20	20	12.7	15.0
6 Basal + 50% alcohol extracted raw beans	6/20	30	7.0	11.0
7 Basal + alcohol extract of raw beans [6]	10/20	50	11.6	11.4
8 Basal + acetone precipitate of alcohol extract (25°) [6]	0/20	0	12.5	12.4
9 Basal + acetone precipitate of alcohol extract (−30°) [6]	2/20	10	9.9	11.4
10 Basal + alcohol extract residue [6]	9/20	45	9.2	11.0
11 Basal + 50% alcohol extracted autoclaved [4] beans	0/20	0	12.9	15.0
12 Basal + alcohol extract of autoclaved beans [5,6]	11/20	55	9.9	10.4
13 Basal + 2.0% linoleic acid	11/20	55	11.0	11.1

[1] Indicates number affected over total number of normal plus affected.
[2] For chicks surviving to the end of the experiment only.
[3] Rovimix E, DL-α-tocopheryl acetate, Hoffmann-La Roche, Inc., Nutley, New Jersey.
[4] At a temperature of 118° for 30 minutes.
[5] At a temperature of 127° for 60 minutes.
[6] All alcohol fractions added to simulate a 100% bean diet.

TABLE 4

Effect of NaOH extraction on PAT [1] activity in kidney beans (trial 3)

Diet	Nutritional muscular dystrophy	
	No. [2]	
		%
1 Basal (with 25 IU of vitamin E [3]/kg of diet)	0/10	0
2 Basal + 50% raw beans	10/10	100
3 Basal + 50% NaOH extracted raw beans	1/10	10
4 Basal + 30% alcohol precipitate of NaOH extract of beans	1/10	10

[1] PAT indicates Phaseolus antagonist to tocopherol.
[2] Refers to number affected over total number.
[3] Rovimix E, DL-α-tocopheryl acetate, Hoffmann-La Roche, Inc., Nutley, New Jersey.

2) reduced the incidence of NMD to 6%. When raw beans were substituted for glucose in the basal diet including 20 IU of vitamin E/kg of diet, the incidence of NMD (diet 3) increased to 86%. Eight of the chicks fed the diet containing 75% raw beans (diet 4) died within the first week; however, 6 of the remaining 8 developed NMD. Autoclaved beans (diets 5 and 6) also decreased the effectiveness of 20 ppm vitamin E in the prevention of NMD. Beans extracted with alcohol (diet 7) also exhibited the anti-vitamin E effect; however, the alcohol extract (diet 8) also had anti-vitamin E activity. A level of 0.3% cystine completely prevented NMD in the negative control diet (diet 9) and in the diet containing 50% raw beans (diet 10).

The chicks fed raw beans or alcohol-extracted raw beans had significantly greater ($P < 0.01$) relative pancreas weights than chicks fed autoclaved beans. These latter chicks had significantly greater relative pancreas weights than chicks fed diets without beans ($P < 0.05$). Wagh et al. (7) also reported that raw beans increased pancreas size in chicks which is apparently a response to the proteolytic enzyme inhibitors. Wagh et al. (7) noted that autoclaving beans at the same pressure and time used in this study completely removed the effect on the pancreas, but the growth performance of chicks fed autoclaved beans was subnormal when compared with that of chicks fed a control diet containing corn and soybean meal. In the experiment reported here all experimental diets produced subnormal growth when compared with a commercial chick diet (diet 11). A level of 75% autoclaved beans decreased growth when compared with the experimental basal diet (diet 2). Raw beans greatly decreased growth when fed at the 50% or 75% levels.

Experiment 2. The results of experiment 2 summarized in table 3 confirm those of experiment 1 with a high percentage of NMD (95%) in chicks fed the negative control diet (diet 1) and the almost complete effectiveness of the addition of 20 IU of vitamin/kg of diet in preventing the occurrence of NMD. Raw beans (diet 3) and beans autoclaved 30 minutes at a temperature of 118° (diet 4) exhibited anti-vitamin E activity. When the added beans were autoclaved for 60 minutes at a temperature of 127° the anti-vitamin E effect was decreased somewhat but apparently still present (diet 5) as judged by the increase in incidence of NMD over that occurring in chicks fed the basal diet. Both the alcohol extract (diet 7) and the alcohol-extracted beans (diet 6) again increased the incidence of NMD over that of the basal group.

The 2 acetone precipitates (diets 8 and 9) from the alcohol extract were ineffective in increasing the incidence of NMD, but the alcohol-acetone soluble residue (diet 10) did exhibit an anti-vitamin E effect. Linoleic acid (diet 13) fed at the level of 2.0% of the diet also increased the incidence of NMD when added to the basal diet. The alcohol extract of the beans (bean fat) was analyzed for fatty acid content by gas chromatography and contained, on a molar basis, 36.3% linolenic, 21.4% palmitic, 21.3% linoleic, 15.2% palmitoleic, 4.3% stearic and 1.5% other fatty acids. Kidney beans contain from 2 to 3% of fat and approximately 50% of this is unsaturated. Therefore, when alcohol extracts were added to the basal diet at 2 or 4 times that present in 50% bean diets the anti-vitamin E effect observed may have been due to the effect of unsaturated fat.

When autoclaved beans, extracted with alcohol, were fed (diet 11) no effect on the vitamin E requirement was noted. The alcohol extract of the autoclaved beans did exhibit an anti-vitamin E effect (diet 12).

These data indicate that 2 factors exist in raw kidney beans which inhibit the effectiveness of vitamin E for prevention of NMD in the chick. One is alcohol-soluble and relatively heat-stable and apparently constitutes the unsaturated fat in the beans. The second factor is not alcohol-soluble and is heat labile. The specific nature of this Phaseolus antagonist to tocopherol (PAT) is unknown at this time. This factor is most probably the factor contributing to earlier results in this laboratory with sheep (1) where autoclaving of beans fed to ewes markedly reduced the NMD incidence in their respective lambs.

Experiment 3. The results are summarized in table 4. Raw beans again ex-

hibited anti-vitamin E activity in the chick but when the beans were treated with 0.2 N NaOH the activity was removed or destroyed. The alcohol precipitate of the NaOH extract did not significantly increase the incidence of NMD.

In all 3 trials the inclusion of raw beans in the diet decreased the activity of vitamin E in preventing NMD in chicks. Some of the anti-vitamin E activity may be explained by the unsaturated fat contained in beans; however, alcohol-extracted beans which contained no measurable amount of ether extract also exhibited anti-vitamin E activity. This non-alcohol-soluble fraction proved to be heat labile.

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Effect of a Folic Acid Antagonist, Aminopterin, on Fetal Development and Nucleic Acid Metabolism in the Rat [1,2]

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ABSTRACT When a single dose of aminopterin was injected into rats on the tenth day of pregnancy, the embryos were destroyed and completely resorbed if the level of dosage was 200 μg or above. Step-by-step reduction of the analogue permitted more of the fetuses to survive and these showed a low number of congenital defects. Fetal liver DNA in these young was depressed at the higher levels of dosage but RNA was unaffected. When folic acid was administered at varying levels one hour prior to an injection of 150 μg aminopterin, the effect of the aminopterin was partially reversed. There was a statistically significant enhancement of the DNA and of the RNA synthesis when the lower levels of folic acid were used. However, at the highest level of folic acid, the DNA and RNA synthesis in this group were both depressed. When folic acid was given alone, the DNA was again significantly depressed at the highest level of dosage but RNA was unaffected. At this higher level of folic acid some abnormal young were found. Thymidine alone or in combination with inosine was unsuccessful in reversing the aminopterin effect.

Early attempts to produce congenital malformations by folic acid deficiency in animals were complicated by concurrent vitamin B_{12} deficiency as Kalter and Warkany (1) pointed out in their review. In 1947, a series of studies by Nelson and coworkers (2–5) began to appear in which congenital anomalies were produced in the rat by folic acid deficiency induced by a crude folic acid antagonist, x-methyl folic acid. The antagonist, aminopterin (4-aminopteroylglutamic acid), has been studied in pregnant rats by Thiersch and Phillips (6) who observed that the fetuses were completely resorbed without the appearance of abnormal young. However, Murphy and Karnofsky (7), Iwahashi (8) and Kotani et al. (9) have reported that some abnormal young were produced when the pregnant rat was treated with aminopterin.

Although inhibition of nucleic acid synthesis has been observed in the nonpregnant rat treated with folic acid antagonists (10-12), very few studies of embryonic synthesis of DNA and RNA have been reported. The most extensive observations of embryonic nucleic acid synthesis are those of Grant (13) in the frog embryo subjected to the folic acid antagonists, amethopterin (4-amino, N^{10}-methylpteroylglutamic acid) and amino-anfol (4-aminopteroylaspartic acid).

The present paper reports the results of a study of fetal development and nucleic acid synthesis in the progeny of rats treated with a single dose of aminopterin, alone or preceded by the administration of folic acid, at a critical stage of pregnancy. Determinations of liver nitrogen, DNA and RNA were carried out in the 21-day-old fetus. Observations of the effects of administration of the ribosides, thymidine and inosine, in conjunction with aminopterin are reported.

EXPERIMENTAL PROCEDURES

Pregnant rats of the Holtzman strain with a known sperm-positive date were used; the sperm-positive date was counted as day zero of the pregnancy. The rats were housed individually in metal cages and the temperature of the room was maintained at 20 to 25°. On days 9 and 10 of the pregnancy, the experimental rats were fed a folic acid-deficient diet [3] with 1% of

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[2] Paper 5406, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[3] Composition of folic acid-deficient diet: (g/100 g diet) dextrose, 53; vitamin-free casein, 22; gelatin, 8; soybean oil, 5; salt mixture USP XIV, 4.3; Ca gluconate, 3; cellulose, 4; L-cystine, 0.45; cholic acid, 0.25; plus vitamin fortification mixture: (g/kg diet) vitamin A concentrate (200,000 units/g), 1.0; vitamin D concentrate (400,000 units/g), 0.06; (in mg/kg) α-tocopherol, 1.0; niacin, 1.0; riboflavin, 0.2; pyridoxine·HCl, 0.2; thiamine·HCl, 0.2; Ca pantothenate, 0.7; biotin, 0.004; and vitamin B_{12}. 0.3.

succinylsulfathiazole added. Stock diet [4] was fed at all other times and food and water were fed ad libitum. Intraperitoneal injections of aminopterin were administered to the animals on the tenth day of the pregnancy and at approximately the same time of day in each case. The aminopterin was dissolved in 0.01 M $NaHCO_3$ and prepared fresh daily. Folic acid [5] was dissolved in 1 M $NaHCO_3$ and injected one hour prior to the aminopterin, since Goldin et al. (14) demonstrated this procedure to be more effective than simultaneous administration. The total volume of each injection did not exceed one milliliter. Thymidine and inosine were dissolved in distilled water and were injected separately either one hour prior to or simultaneously with the antagonist. Untreated animals comprised 2 groups. The larger group was maintained throughout with stock diet, and the smaller group was treated exactly as the experimental animals except that the injection consisted of 0.01 M $NaHCO_3$ only. Twenty three rats remained untreated and served as controls for the study. In the experimental groups, 72 rats were treated with graded doses of aminopterin only, 19 with graded doses of folic acid alone and 46 with both aminopterin and folic acid. An additional group of 46 aminopterin-treated rats were injected with various combinations of thymidine and inosine, and 6 animals were treated with ribosides only.

The rats were killed by chloroform anaesthesia and the pups were removed by caesarian section on the 21st day of gestation. The uterus was inspected for implantation sites and arrested embryos. The surviving fetuses were removed from the uterus and their weight, crown-rump length and apparent abnormalities were recorded. The fetuses were wrapped in foil and frozen immediately. Every effort was made to remove the fetus while it was viable and to allow a minimum of time to elapse between delivery and freezing. At a later date they were removed from the freezer and the fetal livers were extracted, weighed and homogenized in ice-cold saline with a Potter-Elvehjem tissue homogenizer and refrozen immediately. All of the livers from one litter were pooled to prepare one homogenate.

Nitrogen determinations were carried out on diluted samples of the liver homogenates by direct nesslerization according to the method of Lanni et al. (15). The nucleic acids were extracted from the liver homogenates by the Schneider treatment (16) except that perchloric acid was used instead of trichloroacetic acid (17). RNA was determined by the orcinol method of Ceriotti (17) and the color was read at 675 $m\mu$. The indole method of Ceriotti (18) was used for the determination of DNA and the color was read at 490 $m\mu$.

RESULTS AND DISCUSSION

Table 1 presents the data on fetal development when various levels of aminopterin were injected into the rat on the tenth day of pregnancy. With a dose of 200 μg/kg body weight or higher, the pregnancy was interrupted and the embryos were resorbed. Stepwise reduction of the amount of aminopterin injected resulted in an increased number of surviving young in each litter as well as an increase in the number of litters which were maintained, at least in part, for 21 days. The largest dose which can be administered on the tenth day of pregnancy without any apparent detrimental effect for the young rat appears to be 15 μg/kg. Arrested and resorbing embryos appear at levels as low as 30 μg/kg. A very low percentage of congenitally abnormal young were observed. These consisted, on gross inspection, primarily of hydramnios and extreme edema of the fetus. Other anomalies, such as those described by Nelson (3), may have been present but these were not studied in detail since it was necessary to avoid undue delay in freezing the fetus. The weight and crown-rump length of the young from the aminopterin-treated mothers did not differ markedly from that of

[4] Hedin, P. A., and M. O. Schultze 1955 Maternal diet and other factors affecting the lipid content of livers of very young rats. J. Nutrition, 56: 129; contained: (in parts/100) dried skim milk powder, 10; alfalfa leaf meal, 9; soybean meal (expeller type), 9; fish meal (menhaden or sardine), 9; irradiated dried yeast (9000 IU vitamin D/g), 0.01; ground yellow corn, 61.49; and mineral mixture, 1.5. The mineral mixture was obtained from Swift and Company, Chicago, as Swift's All-Purpose Salt Mixture, stated by the manufacturer to contain: ground limestone, rock phosphate, steamed bone meal, molasses, anise, $MgCO_3$, Fe_2O_3, $MnSO_4$, NaCl, KI, $CuSO_4$, and $CoSO_4$. (Calcium, 25.0–27.0%; P, 4.5%; I, 0.003%; NaCl, 16–20%.)

[5] Crystalline folic acid from Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Effect of aminopterin and folic acid on fetal development in the rat

Aminopterin/ kg rat	Folic acid/ kg rat	No. pregnant rats/ group	Mothers carrying young for 21 days	Mean no. arrested and resorbing fetuses/pregnant rat	Mean no. surviving young/pregnant rat	% abnormal [1]
μg	*mg*		%			
Untreated	0	23	100.0	0.0(0–1) [2]	8.6(2–12)	0.0
15	0	4	100.0	0.0 —	9.5(7–12)	0.0
30	0	6	100.0	1.3(0–4)	7.5(5–10)	0.0
50	0	9	100.0	1.4(0–5)	6.3(1–11)	1.9
75	0	7	57.1	2.8(0–8)	1.7(0–7)	1.6
100	0	17	35.5	1.8(0–7)	1.2(0–7)	4.6
150	0	12	18.2	0.8(0–8)	0.5(0–5)	16.6
200	0	9	0.0	0.0 —	—	—
300	0	8	0.0	0.0 —	—	—
100	100	8	100.0	0.0 —	8.0(3–10)	0.0
150	15	10	50.0	2.8(0–9)	2.7(0–9)	1.0
150	75	10	90.0	0.9(0–4)	7.1(0–11)	2.2
150	150	8	100.0	0.8(0–4)	8.2(6–12)	1.4
150	225	10	100.0	1.6(0–8)	8.8(2–11)	1.0
0	15	4	100.0	0.0 —	9.0(7–10)	0.0
0	75	4	100.0	0.0 —	10.5(9–15)	0.0
0	100	3	100.0	0.0 —	10.0(7–13)	0.0
0	150	4	100.0	0.0 —	8.2(5–11)	6.1
0	225	4	100.0	0.0 —	10.0(6–13)	2.5

[1] There were a total of 17 abnormal young, 11 with hydramnios, 3 with edema and 3 with both hydramnios and edema.
[2] Numbers in parentheses indicate range.

TABLE 2

Fetal size in young from rats treated with aminopterin and folic acid

Aminopterin/ kg rat	Folic acid/ kg rat	No. of young	Mean fetal wt	Mean fetal crown-rump length
μg	*mg*		*g*	*cm*
Untreated	0	198	5.2(3.3–6.5)	4.1(3.2–4.9)
15	0	38	5.2(4.0–6.0) [1]	3.9(3.3–4.5)
30	0	45	5.5(4.3–6.2)	4.0(3.4–4.4)
50	0	57	5.1(2.7–6.6)	4.1(3.2–4.6)
75	0	12	4.4(2.1–5.7)	3.8(3.1–4.4)
100	0	20	4.0(2.1–4.8)	3.8(3.0–4.4)
150	0	6	4.1(2.7–4.8)	3.8(3.1–4.4)
200	0	0	—	—
300	0	0	—	—
100	100	64	5.1(3.7–6.1)	4.0(3.4–4.6)
150	15	27	3.9(2.2–5.7)	3.6(2.9–4.3)
150	75	71	4.8(2.6–6.0)	4.0(2.6–4.6)
150	150	66	5.4(4.0–6.6)	4.1(3.1–4.7)
150	225	79	5.6(2.0–6.1)	4.1(3.1–4.6)
0	15	36	4.7(3.8–6.0)	3.8(3.4–4.4)
0	75	42	4.9(3.8–6.4)	4.1(3.0–4.7)
0	100	30	5.2(3.0–5.7)	4.1(3.4–4.5)
0	150	33	5.2(4.0–6.2)	4.2(3.8–4.7)
0	225	40	5.5(4.2–6.2)	4.4(3.9–4.7)

[1] Numbers in parentheses indicate range.

the untreated rats (table 2). Those animals which were treated with an injection of $NaHCO_3$ only, with the folic acid-free diet did not differ essentially from other untreated animals and the results are reported together as untreated. These data agree with those of Murphy and Karnofsky (7) in that very few abnormalities appeared in the young from aminopterin-treated rats. These investigators noted that the fetus either died early or survived the 21 days with no conspicuous developmental anomalies, and that there was a tendency for the entire litter to resorb or to survive and develop normally. However, at the levels of aminopterin used in the present study, there were many instances in which only a fraction of the implantation sites yielded surviving young at 21 days.

Reversal of the toxic effect of aminopterin by graded doses of folic acid administered one hour prior to the aminopterin (at 150 and 100 μg/kg body weight) was studied and these data are also presented in table 1. At a ratio of folic acid to aminopterin of 100:1 (15 mg folic acid/kg), 50% of the litters survived for 21 days but 1% of them were abnormal. An increase in the ratio to 1000:1 (150 mg folic acid/kg) allowed 100% of the litters to survive to 21 days but 1.4% of these young were abnormal. The administration of folic acid in levels 1500 times (225 mg/kg) that of the aminopterin did not result in normal reproduction. The ratio reported by Greenspan et al. (19) which provided complete protection from the lethal effect of aminopterin in mice was much lower (2.5:1). However, Cravens and Snell (20), working with the chick embryo, were unable to reverse the aminopterin-induced depression of chick hatchability with a ratio of folic acid to aminopterin of 50:1.

The possible toxic effects of large doses of folic acid per se cannot be neglected and table 1 presents data on the treatment of pregnant rats with folic acid alone at various levels. The data appear to be comparable with that of the untreated animals except that at the higher levels of 150 and 225 mg/kg abnormal young appeared. Although the numbers of abnormal young were small, such abnormali-

TABLE 3

Effect of aminopterin, thymidine and inosine on fetal development in the rat

Aminopterin/ kg rat	Thymidine/ kg rat	Inosine/ kg rat	No. pregnant rats/ group	Mean no. implantation sites/rat	Mean no. surviving young	Fetal size	
						Mean wt	Mean crown-rump length
μg	*mg*	*mg*				*g*	*cm*
Untreated	0	0	23	8.6	8.6	5.2(3.3–6.5) [1]	4.1(3.2–4.9)
150	7.5	0	4	11.3	0	—	—
150	15.0	0	3	6.7	0	—	—
150	30.0	0	3	9.0	0	—	—
150	60.0	0	4	10.0	0	—	—
150	7.5	15.0	4	8.2	0	—	—
150	15.0	30.0	4	9.2	0	—	—
150	30.0	60.0	4	6.5	0	—	—
150	60.0	120.0	2	9.0	0	—	—
With administration of ribosides one hour prior to aminopterin injection							
150	60.0	0	4	7.2	0	—	—
150	120.0	0	3	7.0	0	—	—
150	240.0	0	4	8.8	0	—	—
150	60.0	120.0	3	10.0	0	—	—
150	120.0	120.0	4	11.5	0	—	—
0	60.0	0	3	9.0	8.0	5.3(4.4–6.1)	4.3(4.0–4.6)
0	240.0	0	1	9.0	9.0	5.5(5.3–5.6)	4.3(3.9–4.4)
0	60.0	120.0	2	8.0	8.0	5.5(5.1–5.9)	4.3(4.1–4.4)

[1] Numbers in parentheses indicate range.

ties have never been observed in untreated rats in this laboratory. It is not possible to determine from these data whether the abnormalities which occurred in the young from rats receiving aminopterin and folic acid were caused by the aminopterin, by the high dose of folic acid or by both. That folic acid is toxic in itself has been reported by Greenspan (19) who observed that folic acid in a dose of 225 mg/kg body weight in mice resulted in the deaths of 4 of 8 mice.

In contrast with the experience of Cravens and Snell (21), with the chick embryo, and Grant (13) with the frog embryo, attempts to reverse the aminopterin toxicity with the ribosides. thymidine and inosine, alone or in combination at various dosage levels, were unsuccessful. These data are presented in table 3. Administration of the ribosides one hour prior to the injection of the antagonist was no more successful than simultaneous administration. Six animals were treated with the ribosides alone and these data were not different from those for untreated animals.

Table 4 presents the results from the determinations of RNA and DNA in the fetal liver homogenates. DNA concentration, expressed as deoxyribose per milligram liver N, decreased with each increment of aminopterin injected into the mother and this decrease approached significance ($P < 0.10$). When both aminopterin and folic acid were administered, there appeared to be an enhancement of the DNA concentration at the 2 lower levels of folic acid (75 and 150 mg/kg). However, at the highest level of 225 mg/kg, there was a statistically significant decrease ($P < 0.05$) in the DNA/mg liver N. This response to increasing levels of folic acid given one hour prior to a constant dose (150 μg/kg) of aminopterin was clearly non-linear (fig. 1), with the maximal response occurring at about 75 mg of folic acid/kg. Tests of significance showed that the response was quadratic

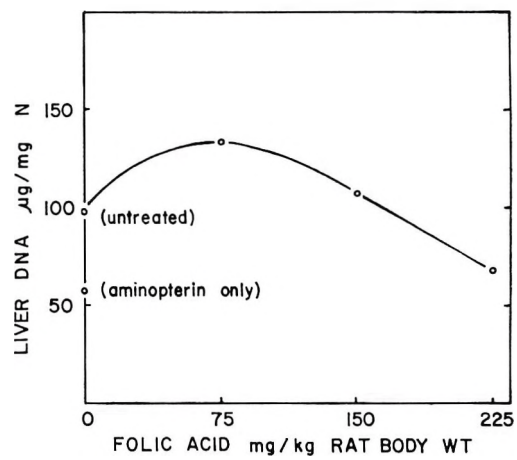

Fig. 1 Liver DNA concentration in young of rats treated with 150 μg aminopterin and 3 levels of folic acid.

TABLE 4

The RNA and DNA content of fetal liver homogenates

Aminopterin/ kg rat	Folic acid/ kg rat	No. pregnant rats/ group	Mean no. livers/ homogenate	DNA (deoxyribose)	RNA (ribose)
μg	*mg*			*μg/mg nitrogen*	*μg/mg nitrogen*
Untreated	0	17	8.2	98.9 ± 7.1 [1]	442 ± 28
50	0	3	9.7	79.2 ± 2.6	491 ± 46
100	0	5	3.2	82.6 ± 12.2	419 ± 33
150	0	1	5.0	57.8 [2]	415 [2]
100	100	4	9.8	110.7 ± 9.1	559 ± 19
150	75	3	6.7	134.0 ± 14.2	495 ± 14
150	150	4	9.2	106.5 ± 13.8	502 ± 19
150	225	7	8.8	67.9 ± 12.9	320 ± 43
0	75	4	10.5	98.4 ± 9.9	359 ± 5
0	150	4	8.2	92.5 ± 16.9	412 ± 26
0	225	4	10.0	56.3 ± 21.5	380 ± 40

[1] SE of mean.
[2] One homogenate only.

($P < 0.02$), indicating that the maximum was undoubtedly a true response. When the mothers were treated with folic acid alone, the DNA concentration was again significantly depressed at the highest level of the folic acid ($P < 0.05$).

There was no significant difference in the RNA concentration (expressed as ribose/mg liver N) in the livers of young from the rats treated with aminopterin alone or with folic acid alone. In the group treated with both aminopterin and folic acid, there appeared to be an enhancement of the RNA which parallels the enhancement of DNA at the 2 lower levels of folic acid and a statistically significant decrease in RNA at the highest level of folic acid ($P < 0.05$). Similarly the response was non-linear (fig. 2) and the tests of

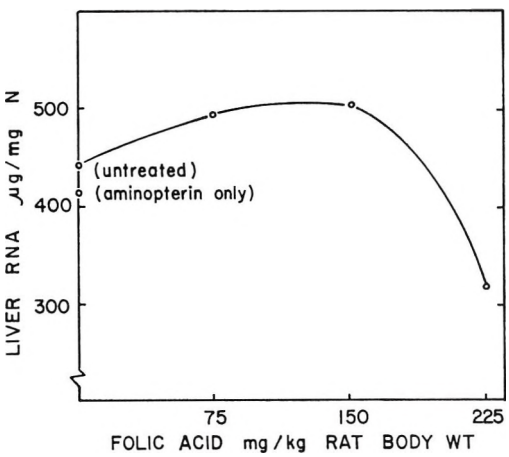

Fig. 2 Liver RNA concentration in young of rats treated with 150 µg aminopterin and 3 levels of folic acid.

significance for the quadratic response ($P < 0.01$) indicated that the maximum was a true response, with the peak occurring in this instance at a level of 150 mg/kg of folic acid.

Decreases in DNA and RNA in selected tissues after treatment of rats with aminopterin have been observed by other workers. Métais (10) observed a decrease in both RNA and DNA concentration in the bone marrow of aminopterin-treated rats. Goldthwait (11) reported that a marked inhibition of formate C^{14} incorporation into the DNA of intestinal mucosa was caused by aminopterin in rats. On the other hand, Guggenheim (22) observed an increase in liver RNA and DNA with aminopterin-treated rats. It is possible that the difference is due to Guggenheim having worked with adult rats, in which the liver may be expected to be less highly mitotic than would be the case with fetal liver. Baron (23), working with the antagonist, amethopterin, reported that DNA synthesis was inhibited in mitotic but not in non-mitotic liver growth. Grant (13) noted in frog embryos treated with amethopterin that the organs with the highest mitotic activity were the most affected by the antagonist, although the cleavage stage was less sensitive to the antagonist than neurulation (lower mitotic stage). He also reported that at doses of amethopterin which inhibited blastulation, the DNA synthesis was unaffected during cleavage but the expected increase of DNA during blastulation leveled off and remained constant, whereas in untreated embryos, DNA continued to increase normally.

Although the depression of DNA observed in fetal liver in these experiments agrees with similar observations of other investigators, it is difficult to explain the enhancement of both DNA and RNA concentration when the pregnant rat is treated with both aminopterin and folic acid.

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Effect of Fasting and Refeeding on Body Weight, Rectal Temperature, Blood Volume and Various Blood Constituents in Growing Swine [1,2]

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ABSTRACT Growing pigs (30-kg) were used to study the effect of fasting and refeeding on body weight, rectal temperature, blood volume and various blood constituents. Nine fasted and 9 control pigs were used for a 167-hour fast and a 216-hour refeeding period. Hematocrit, hemoglobin and serum cholesterol values were significantly greater in fasted pigs after 27 hours. Blood glucose and serum calcium, sodium, urea N, ammonia N, and nonprotein N levels were significantly lower in fasted pigs as compared with control pigs. Fasted pigs had first significantly lower and then significantly higher serum total N and protein levels than control pigs. Blood volume as a percentage of body weight was 53% greater in fasted pigs as compared with control pigs (9.96 and 6.51%, respectively). No significant difference of plasma and total blood volume was observed between control and fasted pigs as determined by the T-1824 dye method. Rectal temperature was significantly decreased when pigs were fasted 27 or more hours. With the exception of hemoglobin, no differences were noted between the fasted and the control pigs at 216 hours after refeeding. Hematocrit and serum total N and protein values from control pigs decreased significantly which suggests that repeated blood sampling may have an effect on these criteria.

Despite homeostasis, blood constituents can be used for evaluating the nutritional and disease status of an animal. In biological research it is important to know the effect of fasting on various blood constituents, and also the length of fast required to get relatively constant values for these constituents. The effects of total and semi-starvation on man and a few other species have been reviewed by Keys et al. (1).

Many workers in the past have observed subnormal body temperatures in starved and undernourished subjects. Excellent reviews of the literature have been made on this subject by Keys et al. (1) and McCance and Mount (2).

Increased hematocrit and hemoglobin values have been observed in partially and completely fasted animals (3–8). In contrast, White et al. (9) reported normal hematocrit and hemoglobin values in sheep fed at decreasing levels of intake followed by a 9-day starvation period, and Keys et al. (1) in man, and McCance (10) in pigs, reported decreased hematocrit and hemoglobin values during semi-starvation.

Although a number of studies of the blood volume of normal swine have been published (11–14), the authors are not aware of any study of the blood volume of fasted swine. Keys et al. (1), in semi-starvation studies with man, demonstrated an increased blood volume as a percentage of body weight but no change in total blood volume.

Nitrogen metabolism plays an important role in all fasting and refeeding studies since a change of nitrogen (N) in the body is associated with a change of living tissue. The magnitude and direction of the change in serum and plasma protein (1, 7, 8, 15–20), blood urea N (21–23) and nonprotein nitrogen (NPN) concentrations (9, 24) during partial and complete fasting have been variable.

The behavior of blood sugar and cholesterol during complete and partial starvation has been variable, with decreased, unchanged and increased levels reported in the literature (1, 22, 24–31). The reports

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of the effect of fasting on blood calcium, sodium and potassium are conflicting (23, 32–36).

The present study was conducted to clarify the effect of fasting and refeeding upon body weight, rectal temperature, blood volume, and various blood constituents.

EXPERIMENTAL

Eighteen 30-kg crossbred pigs about 3 months of age were allotted to 2 equal groups. Sex and weight were balanced. Pigs were housed indoors so that environmental temperature could be controlled within the comfort zone. A dehydrated, ground corn-cob preparation was used as bedding on the concrete floors. Prior to and during the experiment all pigs, except the fasted group, received a 17% crude protein corn-soybean ration fortified with vitamins and minerals (table 1). All

TABLE 1

Composition of ration [1]

	%
Corn	75.30
Soybean meal	20.00
Dehydrated alfalfa meal	2.50
Limestone	0.60
Dicalcium phosphate	0.80
Trace mineral salt (HiZn) [2]	0.50
Vitamin B concentrate [3]	0.10
Vitamin B_{12} supplement (13.2 mg/kg)	0.15
Vitamin A and D mixture [4]	0.05

[1] Contained 17% crude protein by calculation and analysis; also 0.50% calcium and 0.47% phosphorus by calculation.
[2] Contained: (in per cent) cobalt, 0.01; copper, 0.005; iodine, 0.007; iron, 0.15; manganese, 1.2; zinc, 0.8; and salt, 97.
[3] Supplied 4,400 mg riboflavin, 8,800 mg D-pantothenic acid, 19,800 mg niacin and 198,000 mg choline chloride/kg of concentrate.
[4] Contained 7,990,000 IU of vitamin A and 1,760,000 IU of vitamin D/kg.

pigs were offered water ad libitum during the entire experimental period.

Feed was removed from both groups 10 hours prior to and replaced 2 hours before initial blood sampling. After the initial blood samples were taken, feed was removed from one group for the duration of the fasting period which lasted 167 hours. The control group was fed ad libitum.

During the first 24 hours of the refeeding phase, 4 of the fasted pigs were hand-fed 0.22 kg of feed 3 times daily and the other 5 pigs were self-fed. All pigs were fed ad libitum for the remainder of the refeeding phase. Feed consumption of both groups was recorded in the refeeding phase.

Blood samples (17 ml) were taken from the anterior vena cava 7 times during the fasting phase, zero, 6, 15, 27, 51, 95 and 167 hours, and twice in the refeeding phase, 24 and 216 hours. The blood samples were divided as follows: 2 ml were placed in vials containing 1 drop of heparin and 15 ml were placed in centrifuge tubes for clotting. All tubes and vials were tightly corked during all operations. Serum samples were left at room temperature for 1 hour before removal of the clot and centrifugation for 15 minutes at 2000 $\times$ *g*. Hematocrit, hemoglobin and glucose determinations were carried out within 2 hours after blood samples were taken.

Hemoglobin was determined by the cyanmethemoglobin method (37). The hematocrit values were determined by the procedure outlined by McGovern et al. (38). Blood volume was estimated at the end of the fasting phase using the T-1824 dye method described by Gregersen and Rawson (13).

Body temperature (rectal) was determined while blood samples were being taken. Weights were taken at zero, 51, 95 and 167 hours during the fasting phase and at 24, 168 and 216 hours during the refeeding phase.

Total nitrogen in serum samples was determined by a modification of the semi-micro Kjeldahl procedure (39). Brown's paradimethylaminobenzaldehyde method (40) was used for serum urea N and Conway's (41) microdiffusion technique using the Obrink (42) modified Conway dish was used for serum ammonia N. Nonprotein nitrogen was calculated by difference. The serum protein was determined according to the method described by Waddell (43). Electrophoretic separation was accomplished on a Spinco, Model R, paper electrophoresis system [4] at room temperature. The relative intensities of the separated proteins were determined by scanning with a Spinco Model RB Analytrol.

The Nelson-Somogyi (44) microtechnique was used for blood glucose. Serum cholesterol was determined by the method

[4] Spinco Technical Bulletin 6050A, Beckman Spinco, a divisions of Beckman Instruments, Inc., Palo Alto, California.

of Zak as modified by Brown and Wolf (45). Methods of Mori (46) and Appleton et al. (47) were combined and modified for the determination of serum calcium. Serum sodium and potassium determinations were made in a Beckman DU spectrophotometer equipped with a flame attachment using an oxygen-acetylene burner. The data were treated by analysis of variance and correlation analysis (48).

RESULTS AND DISCUSSION

Hematocrit values (table 2) increased significantly after 27 hours of fasting and remained elevated even at 24 hours after refeeding. There was a sharp decrease in the hematocrit of the fasted pigs at 216 hours after refeeding as compared with values at 24 hours after refeeding. This large decline may have been due to the rapid weight gain of these pigs during the 216-hour refeeding phase. The fasted pigs gained 10.5 kg/pig compared with 7.3 kg/pig for the control pigs, with a feed gain ratio of 1.6 for fasted and 2.7 for control pigs. Hemoglobin values of fasted pigs increased as the hematocrit values increased. Hematocrit and hemoglobin values from the initial blood samples are comparable to values reported by Miller et al. (49). Fasted pigs lost an average of 14% of their body weight.

Henderson et al. (4) and Schulz and Muller (5) reported 12 to 13% increases in hematocrit values from rats fasted 4 to 7 days. Body weight loss was approximately 30% in these rats. Schulz and Muller (5) suggested that the hemoconcentration was probably a reflection of the degree of dehydration. A 6-day starvation study carried out by Widdowson and McCance (6) showed that hemoglobin values were increased in fasted rats, and they attributed the increase to a decrease in the blood volume. In contrast, Keys et al. (1), studying men, and McCance (10), growing pigs, reported decreased hematocrit and hemoglobin values under conditions of semi-starvation. White et al. (9) observed normal hematocrit and hemoglobin values in sheep on decreasing levels of feed intake followed by a 9-day starvation period.

The fasted pigs at 15 hours had significantly lower serum total N and serum protein levels than control pigs (table 2). However, at 27 hours for serum protein and 51 hours for serum total N, the levels of these 2 constituents in the fasted pigs were significantly higher than the levels in the control pigs. At 24 hours after refeeding, serum total N levels in the fasted pigs were still significantly higher than control values, whereas serum protein levels were not significantly different. There were no significant differences between groups at 216 hours after refeeding for either constituent.

Decreased serum or plasma protein levels were reported for men semi-starved for 24 weeks (1), chicks starved for 10 days (19), dogs fasted for 3 weeks (15), and rats fasted 8 to 15 days (7, 8, 20). In contrast, no consistent change was observed in the plasma protein of starved dogs (16) or in the serum protein of starved eels (18), whereas increased serum protein was observed in rabbits fasted 5 days (17) and in men fed a semi-starvation diet for 12 weeks (1).

Urea N serum levels of fasted pigs were significantly lower than those of control pigs at 6, 15, 27, 95 and 167 hours (table 2). At 24 and 216 hours after refeeding, urea N levels of fasted pigs were higher than levels of control pigs, but the differences were not significant. Relatively constant blood urea N levels were reported by Baur and Filer (21) in pigs starved until death, and by Sano (18) in eels starved 90 days. Chance et al.[5] observed a decline in plasma urea N levels of swine after 12 hours of fasting and an increase back to near normal after 24 hours. Lennox et al. (22) reported variable changes in the blood urea N levels of fasted men; in some cases there were no changes throughout the fast, whereas in others there was an increase the first week followed by a decline. Increased blood urea N was observed in a man fasted 45 days (23).

Ammonia N serum levels of fasted pigs were significantly less than levels of control pigs at 15, 27, 51, 95 and 167 hours (table 2). The ammonia N levels were not different between groups during refeeding.

Fasted pigs had significantly lower NPN levels than control pigs at 27 and 167

[5] Chance, R. E., W. M. Beeson and E. T. Mertz 1962 Plasma amino acids in swine during growth and fasting. J. Animal Sci., 21: 990 (abstract).

TABLE 2

Body weight, hematocrit, hemoglobin and serum total N, protein, urea N, ammonia N and nonprotein N of fasted and nonfasted pigs [1]

Time, hours	Fasting							Refeeding	
	0	6	15	27	51	95	167	24	216
Body weight, kg									
Control	30.8	—	—	—	33.7	35.2	37.2	37.8	44.3
Fasted	30.0	—	—	—	27.1	26.5	25.6	27.9	36.6
Hematocrit, %									
Control	38.5 ± 0.8 [2]	36.9 ± 0.6	38.7 ± 0.5	34.9 ± 1.1 [3]	35.5 ± 0.8 [3]	35.1 ± 0.8 [3]	35.0 ± 0.9 [3]	34.0 ± 0.6 [3]	36.0 ± 0.7
Fasted	38.6 ± 0.9	37.5 ± 0.7	38.8 ± 0.6	39.1 ± 0.8 [4]	40.0 ± 0.8 [4]	41.7 ± 0.6 [4]	40.4 ± 0.7 [4]	39.5 ± 0.9 [4]	30.7 ± 0.4 [4]
Hemoglobin, g/100 ml									
Control	12.2 ± 0.4	11.8 ± 0.1	12.1 ± 0.2	11.3 ± 0.4	11.5 ± 0.2	10.9 ± 0.4	11.2 ± 0.4	11.5 ± 0.3	10.3 ± 0.2
Fasted	12.6 ± 0.4	12.1 ± 0.2	12.0 ± 0.2	12.3 ± 0.3	13.0 ± 0.4 [5]	13.4 ± 0.2 [4]	13.4 ± 0.3 [4]	13.9 ± 0.3 [4]	10.2 ± 0.3
Total N, mg/100 ml									
Control	1091 ± 19	1083 ± 29	1103 ± 23	1056 ± 18	1033 ± 12 [3]	1017 ± 18 [6]	1026 ± 13 [6]	1009 ± 15	1050 ± 11
Fasted	1076 ± 25	1049 ± 14	1026 ± 12 [5]	1070 ± 15	1093 ± 17 [4]	1103 ± 18 [4]	1078 ± 13 [5]	1071 ± 15 [4]	1035 ± 17
Total protein, g/100 ml									
Control	6.42 ± 0.08	6.29 ± 0.13	6.59 ± 0.18	6.05 ± 0.09 [6]	6.21 ± 0.07	6.11 ± 0.10 [3]	5.99 ± 0.07 [3]	6.01 ± 0.11	6.06 ± 0.06
Fasted	6.37 ± 0.14	6.18 ± 0.10	6.07 ± 0.14 [5]	6.44 ± 0.09 [4]	6.65 ± 0.10 [4]	6.63 ± 0.10 [4]	6.46 ± 0.07 [4]	6.18 ± 0.09	6.01 ± 0.12
Urea N, mg/100 ml									
Control	17.0 ± 1.3	19.1 ± 1.3	18.7 ± 0.7	17.4 ± 0.5	16.2 ± 0.8	17.5 ± 1.1	16.4 ± 1.2	15.1 ± 1.1	17.7 ± 1.2
Fasted	17.6 ± 1.0	13.0 ± 0.7 [4]	11.7 ± 0.8 [4]	13.4 ± 0.6 [4]	14.0 ± 0.9	12.5 ± 1.1 [4]	11.1 ± 0.8 [3,4]	17.7 ± 1.2	19.5 ± 1.2
Ammonia N, μg/100 ml									
Control	845 ± 16	394 ± 6 [3]	472 ± 10 [3]	420 ± 11 [3]	464 ± 11 [3]	542 ± 13 [3]	621 ± 26 [3]	566 ± 9	476 ± 10
Fasted	813 ± 21	485 ± 14 [4]	433 ± 15 [5]	340 ± 16 [4]	418 ± 15 [5]	466 ± 24 [5]	535 ± 13 [3,5]	532 ± 26	480 ± 14
Nonprotein N, mg/100 ml									
Control	64 ± 11	76 ± 13	53 ± 15	87 ± 13	39 ± 6	38 ± 5	66 ± 9	46 ± 8	78 ± 7
Fasted	56 ± 8	59 ± 9	59 ± 19	46 ± 9 [4]	27 ± 4	42 ± 11	44 ± 4 [5]	82 ± 6 [4]	73 ± 9

[1] Nine pigs in both control and fasted groups, except after 6 hours in the fasted group — one pig in this group was accidentally killed during the second bleeding.
[2] SE of mean.
[3] Significantly ($P < 0.01$) different from mean at zero time.
[4] Significantly ($P < 0.01$) different from control mean.
[5] Significantly ($P < 0.05$) different from control mean.
[6] Significantly ($P < 0.05$) different from mean at zero time.

TABLE 3

Serum electrophoretic pattern of fasted and nonfasted pigs

Time, hours	Fasting							Refeeding	
	0	6	15	27	51	95	167	24	216
No. control pigs	9	9	9	9	9	9	9	9	9
No. fasted pigs	9	9	8 [1]	8	8	8	8	8	8
γ-Globulin, %									
Control	20.0 ± 1.0 [2]	20.0 ± 1.4	19.6 ± 1.4	19.8 ± 1.3	19.2 ± 1.5	19.5 ± 1.2	18.3 ± 0.9	18.3 ± 0.9	21.8 ± 1.2
Fasted	19.5 ± 1.5	18.9 ± 1.1	18.9 ± 1.2	19.1 ± 1.1	19.6 ± 1.4	18.4 ± 1.1	16.7 ± 1.1	18.3 ± 0.9	17.8 ± 1.5
β-Globulin, %									
Control	12.0 ± 0.7	11.7 ± 0.9	12.0 ± 0.7	13.0 ± 0.6	13.0 ± 1.1	11.4 ± 0.7	12.1 ± 1.1	12.9 ± 0.7	14.0 ± 0.7
Fasted	13.9 ± 0.9	13.0 ± 0.9	13.8 ± 0.9	12.0 ± 0.6	13.9 ± 0.5	11.3 ± 0.5	11.4 ± 0.5 [3]	13.0 ± 0.8	14.0 ± 0.5
α-Globulin, %									
Control	18.1 ± 0.8	17.7 ± 0.5	17.8 ± 0.8	16.3 ± 0.7	16.4 ± 0.7	17.8 ± 0.9	19.7 ± 1.2	19.6 ± 0.8	17.2 ± 0.9
Fasted	17.8 ± 0.9	17.9 ± 1.1	16.4 ± 0.8	16.9 ± 0.7	15.1 ± 0.8	14.3 ± 0.7 [5]	13.7 ± 0.7 [4,5]	16.2 ± 0.5 [3]	18.6 ± 0.8
Albumin, %									
Control	50.0 ± 1.7	50.5 ± 1.3	50.6 ± 1.8	50.8 ± 1.6	51.4 ± 2.3	51.3 ± 2.1	49.8 ± 2.3	49.3 ± 1.4	47.3 ± 1.9
Fasted	48.7 ± 1.3	50.2 ± 1.1	50.9 ± 0.9	52.1 ± 1.5	51.4 ± 1.2	56.0 ± 1.4	58.3 ± 0.9 [4,5]	52.5 ± 1.5	49.8 ± 1.3

[1] One pig was accidentally killed during the second bleeding.
[2] SE of mean.
[3] Significantly ($P < 0.05$) different from mean at zero time.
[4] Significantly ($P < 0.01$) different from mean at zero time.
[5] Significantly ($P < 0.01$) different from control mean.

hours; however, at 24 hours after refeeding the fasted pigs had significantly higher NPN levels (table 2). These results are in contrast with the continuously increasing blood NPN levels reported for chicks fasted 8 days (24) and with no change in blood NPN levels of sheep fasted 9 days (9).

Gamma and β-globulin levels were not significantly different between fasted and control pigs at any time during the experiment (table 3). Percentage of α-globulin was significantly lower in the fasted pigs than in the control pigs at 95 and 167 hours of fasting and at 24 hours after refeeding. Albumin was increased in the fasted pigs at 95 and 167 hours, but the increase was statistically significant only at 167 hours. The few reports in the literature of the effect of fasting upon the electrophoretic pattern of serum are conflicting. Peterson and Beatty (50) observed no change in the serum protein pattern of rats fasted 24 hours, whereas Krelzchmar [6] reported in mice starved 72 hours a significant decrease in the proportion of α_1- and β-globulin; no change in the proportion of albumin and α_2-globulin; and a barely significant rise in the γ-globulin proportion. Weimer and Nishihara (8) observed a significant decrease in the globulin levels of rats fasted 8 days with no change in the albumin levels.

Blood glucose levels were significantly lower ($P < 0.01$) in the fasted pigs than in the control pigs at 15, 27 and 95 hours (table 4). Levels of blood glucose were also lower in the fasted pigs at 51 and 167 hours; however, the differences were not statistically significant. The glucose level of fasted pigs at 167 hours was significantly less ($P < 0.01$) than the level at zero hours. Further inspection of blood glucose levels of fasted pigs reveals a decrease at 6, 15 and 27 hours and then an increase back to near normal at 51 hours and then another decrease at 95 and 167 hours. This initial decrease during the first part of the fast and then an increase back to near normal has been observed in chicks (24), humans (22), pigs (31) and rats (28). Decreased blood glucose levels have also been observed in fasted sheep (9, 51) and eels (18). On the contrary, increased blood sugar levels were observed in chicks fasted 192 hours (24) and in rabbits fasted 66 hours (31). Unchanged-to-slight decreases in blood glucose levels were reported in fasted dogs (26, 30). There was no significant difference between fasted and control groups at 24 and 216 hours after refeeding.

Serum cholesterol did not increase immediately upon initiation of fasting, but at 27, 51, 95 and 167 hours the levels were significantly higher in fasted than in nonfasted pigs. Level of cholesterol was not significantly different between groups at 24 and 216 hours after refeeding. Increased serum or plasma cholesterol levels have been observed in humans fasted 2 to 7 days (25, 27). Unchanged serum cholesterol levels were reported in rats fasted only 10 to 12 hours (29). In a longer fast with rats (52) and dogs (53), no change was observed in the serum cholesterol levels. Keys et al. (1) concluded that the preponderance of evidence indicates that the concentration of cholesterol in the blood decreases during partial starvation and increases during complete starvation.

Serum calcium levels of fasted pigs were significantly lower than for control pigs at 51, 95 and 167 hours of fasting. Morgulis (34) and Morgulis and Perley (35) observed decreased serum calcium levels in fasted dogs and cats only when the animals had lost from 35 to 43% of their original body weight. Pigs in this study lost only 14% of their body weight. A slight increase in the serum calcium level of a man fasted 45 days was reported by Sunderman (23). Calcium levels were unchanged in rats underfed 7 to 13 weeks so as to lose about 4% of their body weight per week (32). Keys et al. (1) reported decreased plasma calcium levels in men fed semi-starvation diets.

Lower serum sodium levels were observed in the fasted pigs than in the control pigs; however, the differences were not statistically significant throughout the fast. Unchanged serum sodium levels for fasted rats (33) and for fasted dogs (34) and plasma sodium levels for partially starved guinea pigs (36) have been reported.

[6] Krelzchmar, G. 1957 Serumeiweissuntersuchungen an der hungernden Maus. (Studies of serum protein in the starving mouse.) Ztschr. Ges. Inn. Med., 12: 472 (Cited in Nutr. Abstr. Rev., 28: 133, 1958).

TABLE 4

Blood glucose and serum cholesterol, calcium, sodium and potassium levels and temperature of fasted and nonfasted pigs [1]

Time, hours	Fasting							Refeeding	
	0	6	15	27	51	95	167	24	216
Blood glucose, mg/100 ml									
Control	66.5 ± 1.9 [2]	65.2 ± 2.3	72.4 ± 3.0	73.1 ± 3.2	71.9 ± 2.4	70.3 ± 1.5	64.2 ± 2.6	70.7 ± 2.1	66.4 ± 2.0
Fasted	74.1 ± 1.0	66.9 ± 2.9	55.3 ± 3.4 [3]	54.5 ± 1.4 [3]	67.0 ± 2.4	61.5 ± 2.5 [3]	59.4 ± 2.4 [4]	64.0 ± 2.9	69.7 ± 1.8
Serum cholesterol, mg/100 ml									
Control	120 ± 6	116 ± 4	129 ± 6	115 ± 5	115 ± 6	123 ± 6	124 ± 5	107 ± 4	108 ± 4
Fasted	122 ± 5	118 ± 7	128 ± 6	149 ± 6 [3]	197 ± 8 [3]	211 ± 9 [3]	224 ± 18 [3,4]	136 ± 15	109 ± 2
Serum calcium, mg/100 ml									
Control	11.3 ± 0.1	12.0 ± 0.2	11.7 ± 0.4	12.2 ± 0.2	11.8 ± 0.2	12.2 ± 0.2	12.4 ± 0.3 [4]	12.2 ± 0.3	11.8 ± 0.1
Fasted	11.4 ± 0.4	11.9 ± 0.2	11.5 ± 0.2	11.5 ± 0.2	11.3 ± 0.1 [5]	11.2 ± 0.1 [3]	11.1 ± 0.2 [3]	11.9 ± 0.2	12.0 ± 0.3
Serum sodium, mg/100 ml									
Control	312 ± 3	328 ± 3	330 ± 6	302 ± 3	310 ± 2	309 ± 4	312 ± 2	313 ± 3	315 ± 1
Fasted	321 ± 3	308 ± 3 [3]	308 ± 5 [5]	296 ± 4	307 ± 4	302 ± 2	298 ± 2 [3,4]	313 ± 3	313 ± 3
Serum potassium, mg/100 ml									
Control	29.6 ± 0.9	24.7 ± 0.8 [4]	29.1 ± 0.7	24.8 ± 0.8 [4]	23.5 ± 0.4 [4]	24.3 ± 0.5 [4]	25.0 ± 0.5 [4]	24.1 ± 0.5	23.2 ± 0.5
Fasted	25.6 ± 1.1 [5]	25.2 ± 0.7	26.4 ± 1.0	22.9 ± 1.1	24.2 ± 0.6	23.2 ± 1.1	23.3 ± 0.5 [5]	23.3 ± 0.7	24.7 ± 1.2
Temperature, °									
Control	39.9 ± 0.1	39.9 ± 0.1	39.7 ± 0.1	40.0 ± 0.1	40.4 ± 0.1	39.9 ± 0.1	40.0 ± 0.1	39.9 ± 0.1	39.9 ± 0.1
Fasted	39.7 ± 0.1	39.6 ± 0.2	39.2 ± 0.1 [3]	39.0 ± 0.1 [5]	39.6 ± 0.1 [5]	38.9 ± 0.1 [5]	38.6 ± 0.1 [4,5]	38.9 ± 0.1 [5]	39.8 ± 0.1

[1] Nine pigs in both control and fasted groups, except after 6 hours in the fasted group — one pig in this group was accidentally killed during the second bleeding.

[2] SE of mean.

[3] Significantly ($P < 0.05$) different from control mean.

[4] Significantly ($P < 0.01$) different from mean at zero time.

[5] Significantly ($P < 0.01$) different from control mean.

Potassium levels of serum were not consistently lower in the fasted pigs than in the control pigs. Lower serum potassium levels have been reported for fasted dogs (34) and rats (33), whereas unchanged plasma potassium levels were observed for partially starved guinea pigs (36). No explanation is offered for the significant difference of serum potassium between groups at the initial blood sampling period.

The rectal temperature was significantly decreased when pigs were fasted 27 or more hours (table 2). These results agree with semi-starvation data reported by Benedict et al. (54) in humans, McCance and Mount (2) in growing pigs and Morgulis (55) in dogs and with fasting data reported by Morrill (56) in newborn pigs. At 24 hours after refeeding, the temperature of the fasted pigs had not returned to normal, but it was normal at 216 hours after refeeding.

There appeared to be no overeating in this study when fasted pigs were given feed ad libitum. Feed intake was not different between hand-fed and self-fed pigs during the first 24 hours of the refeeding period. Benedict et al. (54) have observed a tendency toward overeating in man following a period of reduced food intake. The general appearance of fasted pigs was good.

Analysis of the blood data from the control group revealed significantly lower hematocrit values and serum total N and protein at 51, 95 and 167 hours than at zero hour (table 2). It is suggested that these decreases are a result of repeated blood sampling. Body stores of erythrocytes and protein should have been large enough to replace these small losses; however, for some unexplained reason, these stores were not sufficiently large, or were not used in this short period of time. It is also possible that the process of taking the blood samples rather than the actual loss of these constituents is responsible for the decreases.

Sugawara (57) reported that repeated bleedings of rabbits caused a marked decrease in erythrocyte count, hematocrit, hemoglobin concentration and total hemoglobin quantity. These rabbits had 20 ml of blood/kg of body weight removed every other day for 15 times. Also, Weimer et al. (58) reported decreased hematocrit values in rabbits which had 8 to 10 ml of blood removed daily for 11 days. The hematocrits values of men phlebotomized in 500-ml amounts weekly decreased from 44 to 35 after 10 weeks (59). In data reported by Chein (60) there was a 3% decrease in hematocrit following the loss of 5 to 10% of the blood volume of the dog. Further work is required to clarify the effect of repeated blood sampling upon various blood constituents.

Blood volume data from pigs at the end of the fasting phase are shown in table 5. Values for control pigs are comparable to values reported by Hansard et al. (11, 12). Plasma and blood volumes were not significantly different between control and fasted pigs as determined by the T-1824 dye method. However, blood volume as a percentage of body weight was 53% greater in fasted pigs as compared with control pigs (9.96% and 6.51%, respectively). Keys et al. (1) working with men receiving a semi-starvation diet (1570 kcal/day) for 24 weeks showed a 20 to 30% increase in blood volume as a percentage of body weight. The subjects of Keys and associates lost 25% of their body weight, whereas animals in this study lost only 14% of their body weight. A constant

TABLE 5

Hematocrit and blood volume data for 8 control and 8 fasted pigs at 167 hours

	Weight	Hematocrit	Plasma volume	Blood volume	Blood volume
	kg	*%*	*%*	*ml*	*% of body wt*
Control	37.2 ± 1.2 [1]	35.0 ± 1.0	1565 ± 63	2426 ± 107	6.51 ± 0.17
Fasted	25.6 ± 0.9 [2]	40.4 ± 0.7 [2]	1511 ± 35	2545 ± 66	9.96 ± 0.23 [2]

[1] SE of mean.
[2] Significantly different from control mean ($P < 0.01$).

blood volume relative to actual body weight has been reported in adult rats starved to death (7).

The plasma and blood volumes (table 5) indicate that there was little or no change in the size of the vascular system under conditions of a 7-day fast. Thus, the increased blood volume as a percentage of body weight in the fasted animals was probably due to a loss of body weight. The effect of fasting on blood volume is, however, confounded by the possible effect of repeated blood sampling. The total volume of blood removed by sampling during the 167-hour fast was approximately 5% of the initial blood volume for all pigs. Examination of the hematocrit data from the control pigs indicates that this removal of blood was compensated for by a positive movement of fluid into the vascular system resulting in a hemodiluting effect.

A correlation analysis of initial weights with each of the criteria at the initial blood sampling (zero time) shows a significant positive correlation for serum total N ($r = 0.52$), protein ($r = 0.48$) and albumin ($r = 0.64$), and a significant negative correlation for serum γ-globulin ($r = -0.48$) and α-globulin ($r = -0.53$). Comparison of the initial weight of the pigs and the change in each criteria during the fast (zero to 167 hours) reveals no significant correlations. Also, there were no significant correlations between weight loss of the fasted pigs during the fast and any of the criteria at the end of the fasting period.

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The Glutamic Acid and Arginine Requirement for High Growth Rate of Rats Fed Amino Acid Diets [1]

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ABSTRACT Rat feeding experiments were conducted to study the influence of glutamic acid and arginine on growth rate, efficiency of food utilization and carcass composition of rats fed amino acid diets. When the concentrations of glutamic acid and arginine were varied simultaneously, results show that each partially supplied the requirement for the other but that both were required in substantial amounts for highest growth rates. Weight gains exceeding 5 g/day were obtained only at dietary levels of 0.8% or more of arginine·HCl and 4% or more of glutamic acid. Glutamine was more effective than glutamic acid but α-ketoglutarate was inhibitory. Diets composed of L-amino acids supported greater weight gains than those containing some racemic mixtures, but did not alter the requirement for glutamic acid and arginine. Performance with the best amino acid diets was equal to that provided by the protein (supplemented gluten) control diet with weight gains of approximately 45 g/week. The evidence is discussed supporting the hypothesis that arginine and glutamic acid function to alleviate the potential ammonia toxicity arising from the feeding of large amounts of amino acids.

It was previously reported (1) that the inclusion of relatively large amounts of glutamic acid in amino acid diets permitted, and in fact appeared to be required for, a high growth rate of rats. At that time it was recognized that the apparent requirement for glutamic acid might be dependent upon the need for arginine. The present paper presents the results of subsequent experiments which demonstrate an interrelationship between the dietary requirements for arginine and glutamic acid and which show glutamine to be even more effective than glutamic acid, promoting a high rate of growth equal to that obtained with protein diets.

EXPERIMENTAL

The influence of arginine on the apparent requirement for glutamic acid was tested by feeding several levels of glutamic acid at each of several concentrations of arginine. Three separate feeding experiments were conducted using 4 basal diets which differed only in their amino acid composition and which were patterned after the mixture used previously (1). Basal diets A and B contained certain amino acids as their racemic mixtures, whereas basal diets C and D contained L-isomers only. For simplicity of presentation the composition of basal diet B is listed in table 1 and the variations describing basal diets A, C, and D are given in the footnotes to the table.

Amino acids were purchased from several suppliers with the stipulation that the quality should meet the specifications and criteria of the National Research Council [2] and that compounds should be chromatographically pure when 100-μg quantities were tested. Prior to acceptance the identity and purity of each lot received was checked in this laboratory by paper chromatography, optical rotation and microbiological assay. The quality was generally very high and the few samples judged to be not acceptable were replaced by the supplier with satisfactory lots.

Experimental procedures. Groups of 5 weanling male rats (Sprague-Dawley or Holtzman) were fed the experimental diets and distilled water ad libitum for 3 weeks. Except in experiment 1, in which the experimental diets were offered on the day the rats were received, the animals were fed an amino acid adaptation diet over a 3-day period prior to dividing them into

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[2] National Research Council, Committee on Biological Chemistry 1960 Specifications and criteria for biochemical compounds, pub. 719. National Academy of Sciences — National Research Council, Washington, D. C.

groups and assigning the experimental diets. The adaptation diet was similar to basal diet B (table 1) and contained 0.81% of arginine·HCl and 5.6% of glutamic acid.

The rats were housed individually in suspended wire-bottom cages in an air conditioned room maintained at 25°. Food intakes (corrected for spillage) and body weights were recorded weekly. At the completion of the feeding period the animals were killed with chloroform, the gastrointestinal contents removed and the carcasses frozen. While still in the frozen state, the carcasses were chopped into small pieces and after mixing with an approximately equal quantity of crushed dry ice, were passed twice through a Hobart grinder. The ground samples were collected in polyethylene bags either individually or by group and were stored at −20° until analysis was performed. Nitrogen was determined in triplicate by the conventional macro-Kjeldahl procedure. Carcass fat was determined by extraction with ether and petroleum ether in Mojonnier flasks after acid hydrolysis as described previously (1). Liver fat was determined for 6 groups of rats by ether extraction in a Goldfisch apparatus after freeze-drying the fresh livers.

TABLE 1

Composition of diets

	%
Amino acid basal diet B [1,2,3]	
L-Histidine·HCl·H_2O	0.675
DL-Isoleucine (50% L-)	1.416
L-Leucine	1.154
L-Lysine·HCl	1.368
DL-Methionine	0.467
L-Cystine	0.332
DL-Phenylalanine	0.838
L-Tyrosine	0.500
DL-Threonine	1.016
DL-Tryptophan	0.215
DL-Valine	1.459
L-Proline	0.392
DL-Alanine	0.437
DL-Aspartic acid	0.704
Glycine	2.009
DL-Serine	0.923
Salts 4 [4]	4.00
Corn oil [5]	5.00
Vitamins [6]	+
Wheat starch [7] to total	100
Gluten control diet	
Gluten [8]	22.515
L-Histidine·HCl·H_2O	0.20
L-Lysine·HCl	1.00
DL-Methionine	0.20
DL-Threonine	0.20
DL-Tryptophan	0.05
Salts 4 [4]	4.00
Corn oil [5]	5.00
Vitamins [6]	+
Wheat starch [7] to total	100

[1] Basal diet A contained 0.556% of urea. Alanine, aspartic acid, glycine and serine were added to make total nitrogen 2.70% of the diet and were varied (in proportions listed) to compensate for nitrogen contributed by arginine and glutamic acid in the experimental diets.

[2] Basal diet C contained only L-amino acids in the same amounts as listed.

[3] Basal diet D contained only L-amino acids as in basal diet C but with one-half the listed percentages of isoleucine, threonine and valine.

[4] Salt mixture: Hegsted et al. 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138*: 459; obtained from Nutritional Biochemicals Corporation, Cleveland.

[5] Mazola, Corn Products Company, Argo, Illinois.

[6] Vitamins were supplied in a portion of the starch in mg/100 g of diet as follows: thiamine·HCl, 1; riboflavin, 1; pyridoxine·HCl, 1; nicotinic acid, 10; *i*-inositol, 20; *p*-aminobenzoic acid, 20; folic acid, 0.1; biotin, 0.1; menadione, 2.0; Ca pantothenate, 4; choline chloride, 150; vitamin B_{12}, 0.004. Each rat received weekly 2 mg α-tocopheryl acetate dissolved in 2 drops of corn oil. Vitamins A and D were supplied by a drop of cod liver oil concentrate given to each rat weekly.

[7] Aytex P, General Mills, Minneapolis.

[8] Contained 12.0% of nitrogen.

RESULTS

Experiment 1. Increments of arginine and glutamic acid were added to basal diet A in the amounts shown in table 2. In this experiment only, dietary nitrogen was maintained constant at 2.70% by adjusting the levels of a mixture of alanine, aspartic acid, glycine and serine having the proportions (relative to one another) shown in table 1.

The results of experiment 1 (table 2) demonstrate that weight gain, nitrogen gain and food efficiency tended to vary with the level of both arginine and glutamic acid. At a given level of either of the test compounds, increments of the other yielded increased response up to the highest level tested. The apparent requirement for either arginine or for glutamic acid thus depends upon the concentration of the other. Relatively large amounts of both amino acids were required for maximal rate of gain. In this experiment the greatest growth rates were supported by the combination of 4.23% of glutamic acid with 1.62% of arginine·HCl and by 8.47% of glutamic acid with 0.81% of arginine·HCl. These results are in accord with those of a previous study (1) in which it was found that 5.6% or more

of glutamic acid was required for maximal gain at an arginine·HCl concentration of 0.81%.

The possibility was considered that the apparent requirement for glutamic acid and the need for a large amount of arginine might be related to the large amount of nonessential nitrogen contained in the basal diet. If so, some of the effects attributed to added arginine and glutamic acid might have resulted from the simultaneous decrease in the amounts of alanine, aspartic acid, glycine and serine in this experiment. Subsequent experiments were designed to eliminate such a possible effect by using basal diets of constant amino acid composition to which increments of arginine and glutamic acid were added without compensating for their nitrogen contribution.

Experiment 2. Increments of arginine and glutamic acid were added to basal diet B in the amounts indicated in table 3 for diet groups B-1 through B-12. The levels used in diets B-1 and B-7 were also tested with the L-amino acid basal diets C and D (diets C-1, C-7 and D-1, D-7, respectively) to determine whether the requirement for arginine and glutamic acid would be altered in the absence of D-isomers. As indicated in table 1, basal diet C was identical to basal diet B except that the same percentages of L-amino acids were substituted for the racemic mixtures. Basal diet D was formulated similarly, but with one-half the amounts of L-isoleucine, L-threonine and L-valine used in basal diet C to avoid the large excess of those amino acids whose D-isomers are poorly convertible.

The results obtained with basal B diets in table 3 show that weight gain, food efficiency and carcass nitrogen gain tended to be increased by the additions of arginine and of glutamic acid, as was also observed in experiment 1 (table 2). Although comparisons between experiments are limited because of differences in the levels tested, the stimulatory effects of comparable increments of arginine and of glutamic acid were nearly equal in experiments 1 and 2, indicating that little if any effect could be ascribed to the reduction of nonessential amino acids in basal diet A of experiment 1. For example, at 4% of glutamic acid (4.23% in experiment 1) increasing the arginine·HCl in the diet from 0.41 to 1.62% resulted in increased weight gains of approximately 10 g/week in both experiments; at the level of 0.41 of arginine·HCl, increasing the glutamic acid in the diet from 2 to 8% (2.12 to 8.47% in experiment 1) increased the weight gains by nearly 6 g/week in both experiments. The highest growth rates with basal diet B occurred with the combination of 4% of glutamic acid with 1.62% of arginine·HCl and 8% of glutamic acid with 0.81% of arginine·HCl, in agreement with the observations for basal diet A of experiment 1.

TABLE 2

Effect of additions of glutamic acid and arginine to basal diet A (constant total nitrogen),[1] *fed for a 3-week period*

Diet group	Glutamic acid	Arginine·HCl	Avg wt gain	Food efficiency	Avg N gain[2]	Avg carcass[3]	
						Nitrogen	Fat
	%	%	*g/week*	*g gain/g food*	*g/week*	%	%
A-1	0	0.81	15.2	0.35	0.40	2.75	7.9
A-2	0	1.62	16.7	0.36	0.46	2.82	7.8
A-3	0	3.24	27.9	0.48	0.74	2.82	10.0
A-4	2.12	0.41	23.1	0.45	0.60	2.75	7.7
A-5	4.23	0.41	26.3	0.46	0.70	2.76	10.7
A-6	4.23	0.81	30.8	0.50	0.81	2.80	11.2
A-7	4.23	1.62	36.5	0.49	0.98	2.81	10.2
A-8	8.47	0	15.7	0.35	0.43	2.80	11.4
A-9	8.47	0.41	28.6	0.48	0.77	2.79	10.0
A-10	8.47	0.81	36.4	0.48	1.00	2.85	8.8
Gluten control diet			45.4	0.53	1.32	2.95	12.1

[1] See footnote 1, table 1.
[2] Increase in carcass nitrogen over control group killed on first day.
[3] Determined on carcass after removal of gastrointestinal contents, results expressed on fresh-weight basis.

TABLE 3

Effect of additions of glutamic acid and arginine to a constant level of 3 basal amino acid mixtures,[1] fed for a 3-week period

Diet group	Glutamic acid	Arginine·HCl	Avg wt gain	Food efficiency	Avg N gain [2]	Avg carcass [3]	
						Nitrogen	Fat
	%	%	*g/week*	*g gain/g food*	*g/week*	%	%
B-1	2.0	0.20	24.5	0.38	0.69	2.85	8.7
B-2	2.0	0.41	28.4	0.44	0.77	2.83	7.3
B-3	2.0	0.81	30.3	0.43	0.82	2.86	7.1
B-4	2.0	1.62	29.1	0.42	0.82	2.95	8.0
B-5	4.0	0.20	28.3	0.39	0.69	2.82	9.8
B-6	4.0	0.41	27.6	0.42	0.80	2.95	8.2
B-7	4.0	0.81	32.4	0.44	0.91	2.92	8.2
B-8	4.0	1.62	37.7	0.47	1.05	3.01	9.2
B-9	8.0	0.20	27.7	0.41	0.76	2.83	10.0
B-10	8.0	0.41	34.1	0.48	0.96	2.94	8.6
B-11	8.0	0.81	35.1	0.48	1.00	2.98	8.3
B-12	8.0	1.62	32.5	0.45	1.05	2.99	10.9
C-1	2.0	0.20	28.3	0.40	0.77	2.83	10.9
C-7	4.0	0.81	35.1	0.48	0.98	2.92	9.2
D-1	2.0	0.20	28.3	0.39	0.77	2.83	11.0
D-7	4.0	0.81	35.8	0.44	1.06	3.01	8.3
Gluten control diet			46.0				

[1] Letter prefix of diet group refers to basal diet fed.
[2] Increase in carcass nitrogen over control group killed on first day.
[3] Determined on carcass after removal of gastrointestinal contents, results expressed on fresh-weight basis.

Diets prepared with basal diets C and D were equal in effect to each other at each of the 2 combinations of arginine and glutamic acid tested (diets C-1 vs. D-1 and C-7 vs. D-7). They were superior to the corresponding diets containing basal diet B (diets C-1 and D-1 vs. B-1; and C-7 and D-7 vs. B-7) as shown by all criteria of performance (table 3). The better performance with the L-amino acid diets is not interpreted as evidence for a reduced requirement for either arginine or glutamic acid, or both, because the higher level of these substances produced equivalent improvement in response regardless of the presence or absence of D-amino acids (diets B-1 vs. B-7; C-1 vs. C-7 and D-1 vs. D-7).

Basal diets C and D differed from basal diet B in influencing the deposition of liver fat. Postmortem examination revealed visible mottling in livers of all animals that had been fed the lowest levels of arginine and glutamic acid with the L-amino acid basal diets (diets C-1 and D-1) but not in those of rats fed basal diet B at the same rate of addition (diet B-1). Analyses for liver fat confirmed the visual observations, as shown in table 4. Livers of rats that had consumed the higher amounts of arginine and glutamic acid were normal in appearance and fat content regardless of the basal diet used (groups B-7, C-7 and D-7).

Experiment 3. The ability of related substances to replace glutamic acid was tested with basal diet D in the presence of 0.81% of arginine·HCl. The compounds tested and the levels fed are shown together with the results in table 5. The data for glutamic acid (diets D-9 through D-13) confirm the superior growth rate supported by the L-amino acid basal diet noted in experiment 2. Glutamine was more effective than glutamic acid at equimolar concentrations except at the highest level of glutamic acid used. Values for weight gain, food efficiency and nitrogen gain at the highest levels fed (diets D-16—D-18) were equal to those obtained with the gluten control diet and exceed any obtained to date with other amino acid diets. α-Ketoglutaric acid was not only less effective than glutamic acid but was inhibitory at the higher level. Monosodium glutamate was used to about the same extent as glutamic acid although the higher level produced no increase in gain over that obtained with the lower level.

The data for carcass composition in tables 2, 3, and 5 show that the gains in

TABLE 4

Liver fat content of rats fed at 2 levels of arginine and glutamic acid with basal diets B, C, and D (from table 3)

Diet group [1]	Glutamic acid	Arginine·HCl	Avg wt of liver	Liver fat
	%	%	g	% wet wt
B-1	2.0	0.20	8.0	2.6
C-1	2.0	0.20	8.8	5.7
D-1	2.0	0.20	9.9	5.1
B-7	4.0	0.81	9.4	1.4
C-7	4.0	0.81	9.1	1.3
D-7	4.0	0.81	10.2	1.5

[1] Letter prefix of diet group refers to basal diet; see table 1 for description.

TABLE 5

Effect of additions of glutamic acid and related compounds to a constant level of basal diet D,[1] fed for a 3-week period

Diet group	Test substance	Level [2]	Avg wt gain	Food efficiency	Avg N gain	Avg carcass	
						Nitrogen	Fat
		%	g/week	g gain/g food	g/week	%	%
D-9	Glutamic acid	2.00	36.5	0.45	1.01	2.89	10.2
D-10		3.00	35.5	0.46	1.01	2.94	9.2
D-11		4.00	38.6	0.46	1.09	2.94	— [3]
D-12 [4]		5.00	38.5	0.47	1.10	2.92	10.0
D-13		6.00	44.4	0.49	1.27	2.97	9.7
D-14	Glutamine	1.99	39.3	0.48	1.12	2.93	10.4
D-15		2.98	41.7	0.49	1.16	2.93	9.9
D-16		3.97	45.0	0.48	1.29	2.96	9.6
D-17		4.97	44.4	0.48	1.31	3.01	10.6
D-18		5.96	45.7	0.49	1.33	2.97	10.5
D-19	α-Ketoglutaric acid	1.99	31.7	0.44	—	—	—
D-20		4.97	26.9	0.37	—	—	—
D-21	Monosodium glutamate	2.54	37.3	0.45	—	—	—
D-22		6.36	37.3	0.47	—	—	—
Gluten control diet			45.6	0.51	1.35	3.01	10.3

[1] All L-amino acids (table 1) plus 0.81% of arginine·HCl.
[2] Related compounds provided in equimolar amounts, relative to glutamic acid as follows: diets D-14 — D-18, as in diets D-9 — D-13; diets D-19 and D-21, as in D-9; diets D-20 and D-22 as in D-12.
[3] Analyses not performed.
[4] Group D-12 contained 10 animals.

weight represented deposition of protein and not merely accumulation of fat. The variation in carcass composition was related to the amounts of arginine and glutamic acid in the diet but not to the basal diet used. Carcass nitrogen tended to increase with additions of both arginine and of glutamic acid. Carcass fat tended to increase with glutamic acid but for a given level of the latter, it was less at 0.4 and 0.8% of arginine·HCl than at the other levels fed.

DISCUSSION

The essentiality of glutamic acid and its relationship to the requirement of the rat for arginine was first studied by Rose and co-workers. Womack and Rose (2) showed evidence for the interconversion of arginine, glutamic acid and proline and noted that the addition of arginine alone to an arginine-free amino acid diet was not as effective as when added in combination with glutamic acid. At that time they considered the possibility that glutamic acid was essential, in the sense that its presence was required for rapid growth rate, but tentatively regarded it to be nonessential because it was less effective than arginine when added singly to a diet devoid of arginine and because diets containing 2% of glutamic acid were improved by the addition of arginine. Subsequently (3) it was reported that omission of the 2% of

glutamic acid from a mixture of 19 amino acids caused a reduction in weight gain which was of doubtful statistical significance and on this basis, classified glutamic acid as nonessential for the rat.

It is not the purpose of this discussion to question whether glutamic acid should be labeled as "essential" or "nonessential" since it is generally understood that the distinction is a matter of definition. But it is believed that attention should be called to evidence which suggests that under certain conditions at least, a dietary requirement exists for glutamic acid (or glutamine) which appears to be distinct from its supplementation of arginine.

The data of Womack and Rose (2) show that the addition of 2% of glutamic acid produced approximately the same increase in weight gain with 1.1% of arginine in the diet as with lesser amounts of arginine. The data of table 2 in the present report show that in the absence of glutamic acid in the diet, growth was progressively stimulated by additions of arginine, but was still below the maximal rate of gain even at the level of 3.2% of arginine·HCl. An elevated requirement for arginine with a dependence upon glutamic acid was also observed by Ramasarma et al. (4) who reported that 2% of glutamic acid together with a mixture of nonessential amino acids produced a higher growth rate than when glutamic acid served as the sole source of nonessential nitrogen. Moreover, when the arginine·HCl concentration was increased from 0.25 to 0.75% a significantly greater rate of growth was obtained with the former diet but not with the latter. If glutamic acid acts only to serve as a source of arginine, as suggested by Rose's conclusions, it would be expected that in the presence of a sufficient quantity of arginine no need for glutamic acid would be demonstrated. All of the above observations would be more consistent with the hypothesis that a dietary requirement exists for both arginine and glutamic acid; that through interconversion each is capable of supplementing moderate deficiencies of the other, but for the highest rates of gain the requirement for each must be supplied in the diet.

The results of experiments reported here permit an estimate of the individual requirements for arginine and glutamic acid. Growth rates exceeding 5 g/day were achieved only with those diets which contained 4% or more of glutamic acid and the greatest weight gain occurred with 6%. Ramasarma et al. (4) attributed their failure to obtain increased growth response to additions of glutamic acid greater than 2% to the fact that such additions were made at the expense of the entire nonessential amino acid mixture. In the present studies glutamic acid was added either without reduction of other nitrogenous components (tables 3, 4) or at the expense of a mixture of alanine, aspartic acid, glycine and serine (table 2).

With respect to arginine, the data show that weight gains exceeding 5 g/day were supported only by concentrations of at least 0.8% (as the hydrochloride) which approaches the arginine requirement value of 0.77% of the diet calculated by Williams et al. (5) from the relative proportions of amino acids in rat carcass. Ramasarma et al. (4) concluded from their work that the dietary requirement of 0.2%, cited by Borman et al. (6), may be insufficient with diets that permit a higher growth rate. It is doubtful that rapid growth in itself creates the increased demand, however, because high rates of gain were reported by Rama Rao et al. (7) with diets containing only 0.2% arginine. Also, in the present study, although the different basal diets permitted a range of maximal growth rates, the amounts of arginine and glutamic acid required to attain these gains were similar with each basal diet.

The demand for arginine and glutamic acid probably depends upon the amino acid mixture of the diet. Glutamic acid has been shown to be less effectual as the sole source of nonessential nitrogen than when fed with a mixture of nonessential amino acids (1, 4), which may explain the observations of others that glutamic acid is no more effective than (8), or only slightly superior to (9) other single sources of nonessential nitrogen. Similarly, Winitz et al. (10) showed that the growth response to arginine increased with the complexity of the nonessential amino acid mixture. The above observations suggest that the conditions for obtaining high rates of gain with amino acid diets require that the diet con-

tain substantial amounts of arginine and glutamic acid or glutamine, plus a complete or nearly complete mixture of nonessential amino acids in addition to the known requirements. These observations may not prove, but are consistent with, the hypothesis that arginine and glutamic acid are concerned with the disposition of potentially toxic amounts of ammonia arising from the feeding of large quantities of amino acids.

In a series of reports, summarized by Greenstein and Winitz (11), Greenstein and co-workers demonstrated that intraperitoneal injections of arginine or of arginine plus glutamate effectively reduced the toxicity of injected ammonium salts or amino acids, lowering the ammonia content of the blood and increasing the production of urea. Orally administered arginine was much less effective (12). Kamin and Handler (13) showed that the greatest rate of urea formation occurred upon administration of both arginine and glutamine. Later work from this group (14) showed that the formation of glutamine was a major means of maintaining a low level of ammonia in blood and body tissues and that the rate of urea formation depended both upon the rate of synthesis and the rate of transport of substrate ammonia to the site of urea-synthesizing enzymes. Their results showed that the latter process constitutes the rate-limiting step in urea formation.

The inhibitory action of α-ketoglutarate (table 4) supports the above hypothesis because, as shown by the in vitro work of Ratner and Pappas (15), this compound inhibits urea formation by influencing the transaminase equilibrium in the direction unfavorable to the formation of aspartate.

The relatively high requirement for glutamic acid and the greater effectiveness of glutamine may be explained by their different rates of permeability. Fridhandler and Quastel (16) demonstrated that glutamic acid was slowly absorbed from the intestines and a high concentration was necessary for it to be absorbed unchanged. Glutamine, on the other hand, was absorbed rapidly. Kamin and Handler (13) related the failure of glutamic acid to promote rapid urea formation in vivo to the slow rate of its penetration into liver cells.

The relatively greater activity of glutamine as compared with that of glutamic acid leads to the question of whether the destruction of amide nitrogen during hydrolysis might explain the failure of acid hydrolyzates to provide as good growth as the untreated protein. Although the investigations of Woolley on strepogenin implicated glutamine and certain peptides of glutamic acid (17, 18), very few animal feeding studies have touched on this area. Thorough investigations on the possible influence of glutamine and asparagine on the growth of animals fed synthetic diets are needed to answer this question.

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