Gross and Microscopic Pathology of Tocopherol-deficient Mink[1]

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ABSTRACT Experimental tocopherol deficiency was produced in young male mink to study the subsequent gross and microscopic lesions and the extent to which they were altered or prevented by the addition of cod liver oil, selenium or α-tocopherol to the basal ration. The major gross lesions consisted of internal intercostal, adductor and cardiac myopathy, hepatic peripherolobular fatty infiltration and, in the cod liver oil-supplemented mink, yellow fat. Histologically, the tocopherol-deficiency myopathy was characterized by swollen, differentially stained fibers, vacuolar degeneration, sarcolemmal and myoblastic proliferation and calcification of the nonphagocytized myofibrillae. The myocarditis was focal calcified necrotic and the fatty hepatic infiltration was accompanied by centrolobular hepatic hemorrhage. Yellow, acid-fast pigment was deposited at the interstices of the adipose tissue from the cod liver oil-supplemented mink which also had coagulation necrosis and calcification of the renal tubules. Adipose tissue from chronically tocopherol-deficient mink had adipocyte-like, non-acid-fast spheres in its interstices. The basal tocopherol-deficient ration with 0.1 ppm of selenium as sodium selenite prevented all lesions except minor accumulations of amorphous non-acid-fast material at the adipose interstices. The basal mink ration with 25 ppm α-tocopherol prevented all the tocopherol deficiency lesions.

The limited information available relative to tocopherol deficiency of mink has been obtained since 1947 when McDermid and Ott (1) first reported natural cases of steatitis or yellow fat in mink in the United States. Hartsough and Gorham (2) considered this steatitis to be histologically similar to that experimentally produced by Dam (3) in rats fed low tocopherol, cod liver oil-supplemented rations. The prophylactic effects of tocopherol supplementation of various mink rations were then studied by numerous investigators including Lalor et al. (4) and Dalgaard-Mikkelsen et al. (5). Even synthetic antioxidants such as nn′-diphenyl-*p*-phenylenediamine (DPPD) and butylated hydroxytoluene (BHT) were found effective in preventing steatitis by Leekly and Cabell (6). Zenker's degeneration or "white heart" disease was then reported by Benson (7) to be common in older mink and tocopherol supplementation of the mink rations provided effective prevention of the cardiac degeneration.

During an investigation relative to the possible relationships between an experimental tocopherol deficiency and urinary incontinence, "wet belly," in mink, Stowe et al.[4] found gross evidence of tocopherol deficiency myopathy. The purpose of this paper is to present the gross and microscopic lesions observed during 2 subsequent studies of tocopherol-deficient mink.

EXPERIMENTAL

Experiment 1. Forty, 6-week-old, dark, male, mink kits were fed the semipurified tocopherol-deficient basal ration described for experiment 1 in table 1. The amino acid and vitamin supplement for this ration was prepared to furnish the constituents at the rates shown in table 2. All

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[4] Stowe, H. D., C. K. Whitehair and H. Travis 1960 Pathology of vitamin E deficiency in mink. Federation Proc., *19:* 420 (abstract).

TABLE 1

Composition of the tocopherol-deficient mink diets

	Exp. 1	Exp. 2
	%	%
Vitamin-free casein	16.0	8
Isolated soy assay protein[1]	8.0	16
Torula yeast[2]	16.0	20
Sucrose	25.5	26.5
Molecularly distilled lard[3]	24.0	20.0
α-Cellulose[4]	6.0	5.0
Salt mix (IV)[5]	4.0	4.0
Amino acid and vitamin supplement	0.5	0.5

[1] From Archer-Daniels-Midland Company, Cincinnati, Ohio.

[2] Furnished by Lake States Yeast and Chemcial Division of St. Regis Paper Company, Rhinelander, Wisconsin.

[3] Furnished by Distillation Products Industries, Rochester, N. Y.

[4] Solka Floc, Brown Company, Boston 14, Mass.

[5] Phillips and Hart 1935 J. Biol. Chem., *109*: 657.

TABLE 2

Amino acid and vitamin supplementation rate for experimental mink diets

	Rate[1]
Arginine·HCl	250.0
DL-Methionine	100.0
Riboflavin	1.0
Pyridoxine	0.5
Ca pantothenate	3.6
Nicotinic acid	5.0
Choline chloride	400.0
Thiamine·HCl	0.5
Inositol	50.0
p-Aminobenzoic acid	100.0
Folic acid	0.2
Cyanocobalamin	0.16
Biotin	0.05
Vitamin A acetate	0.52
Vitamin D_2	0.01
Menadione	0.5

[1] Mg/100 g dry diet.

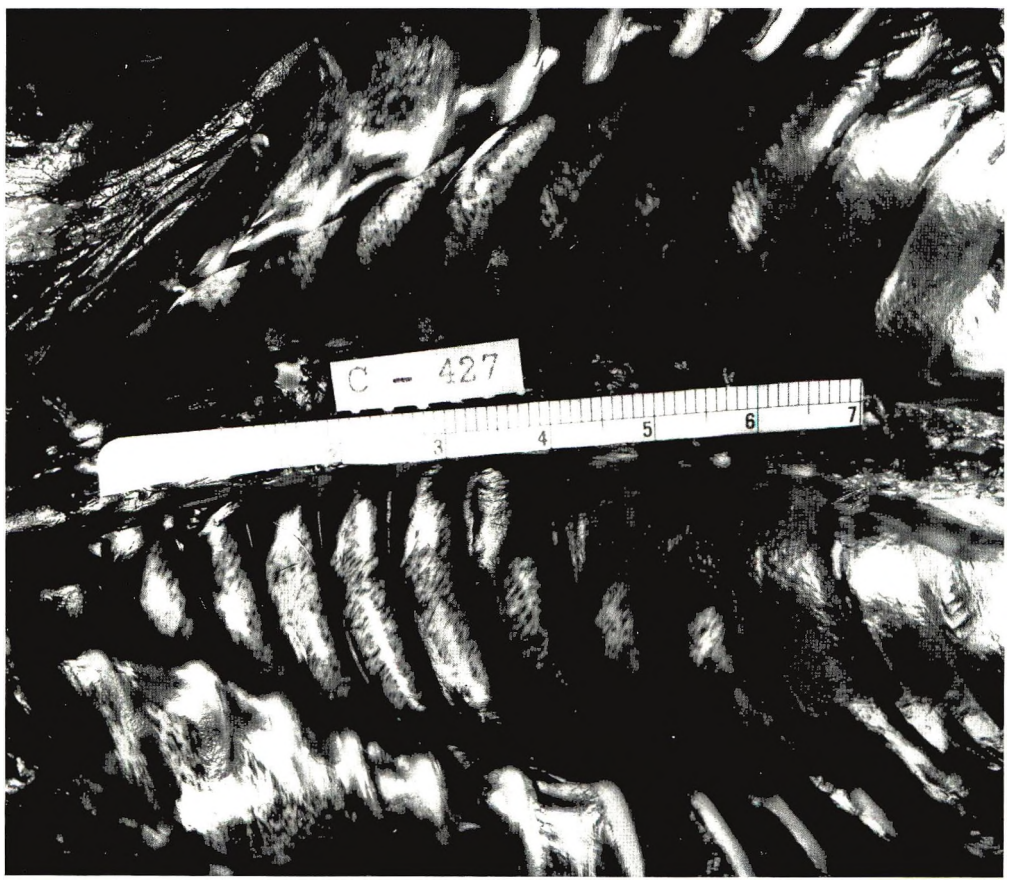

Fig. 1 Bilateral internal intercostal myopathy in a tocopherol-deficient mink.

the animals were maintained with the basal ration for 4 weeks after which 8 mink were assigned to lot A, and their basal ration was supplemented with α-tocopherol at the rate of 150 ppm on a dry basis. When sudden deaths became frequent among the tocopherol-depleted animals, lots B, C and D were established with 8 animals/lot. The basal ration for lots B and C was supplemented with α-tocopherol at the levels of 50 and 25 ppm, respectively. The basal ration for lot D was supplemented with sodium selenite at the level of 1 ppm. The unallotted mink were used as replacements.

Experiment 2. Thirty, 9 - week - old, brown, male, mink kits were fed the semipurified tocopherol-deficient ration shown for experiment 2 in table 1. When the mink became fully accustomed to the ration, they were allotted to one of the following experimental lots: (A) Basal tocopherol-deficient ration; (B) basal with 8% cod liver oil replacing an equal quantity of lard; (C) basal plus 0.1 ppm selenium; and (D) basal plus 25 ppm α-tocopherol.

Mink that died or that were killed at the termination of each experiment were examined by standard necropsy procedures. Sections from the following organs were preserved in acetate-buffered 10% formalin and Zenker's fixatives for histological examination; internal intercostal, adductor magnus and cardiac muscles, diaphragm, trachea, lung, parotid salivary

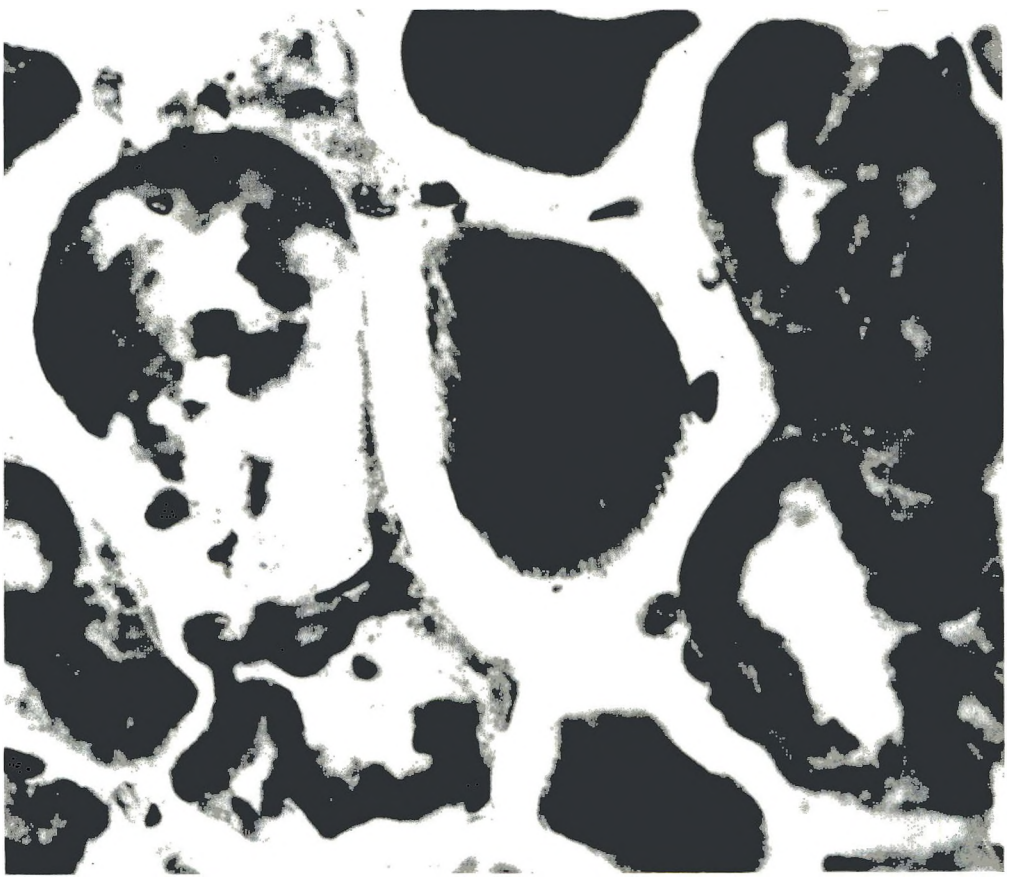

Fig. 2 Vacuolar degeneration of internal intercostal muscle fibers from a tocopherol-deficient mink. H & E stain × 660.

gland, small intestine, pancreas, liver, kidney, adrenal gland, ureter, urinary bladder, urethra and any other tissue considered important at the time of necropsy.

Appropriate sections of each of the tissues were stained with hematoxylin and eosin, Von Kossa's, periodic acid Schiff, Sudan IV and Ziehl-Neelsen stains. The histological procedures followed were according to the Armed Forces Institute of Pathology Manual of Histological and Special Staining Technics (8).

RESULTS

The major lesions of tocopherol-deficient mink included skeletal and cardiac myopathy, steatitis, hepatopathy and nephropathy. In addition, less frequently observed lesions involved the adrenal gland. Lesions were not found in the sections saved from other organs.

Myopathy. Skeletal myopathy was observed grossly and involved primarily the internal intercostal muscles (fig. 1) and the insertion end of the adductor muscles. The myopathy was usually bilateral; however, some unilateral lesions were observed.

Histopathologically, 3 phases of skeletal myopathy were observed. The first phase was characterized by swollen, differentially stained muscle fibers, vacuolar degeneration (fig. 2) or an increased number of irregularly spaced sarcolemmal nuclei, or both. In the second phase, illustrated in figure 3, the previous features were present

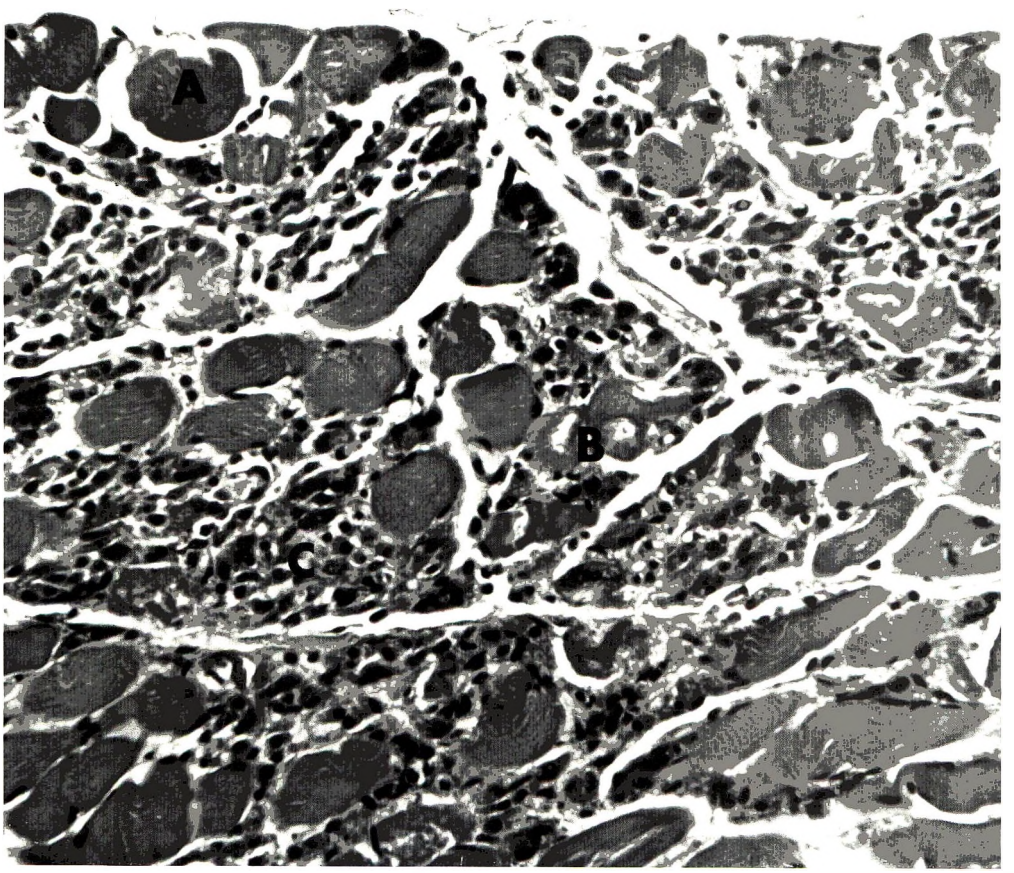

Fig. 3. Adductor from a tocopherol-deficient mink; (A) Swollen, differentially stained fibers; (B) vacuolar degeneration; and (C) proliferating sarcolemmal cells and myoblasts. H & E stain × 350.

along with an extensive nuclear element composed primarily of sarcolemmal nuclei and myoblasts. Lymphocytes and myophages were often present. The third or reparative phase was characterized by focal calcium deposits adjacent to normal muscle fibers (fig. 4). Occasionally the myophagic reaction and calcification of individual myofibrils shown in figure 5 were observed.

Although gross evidence of myopathy was more frequent in the internal intercostal muscles, microscopic lesions were observed with greater frequency in the adductor muscles. The increase in sarcolemmal nuclei was more prominent in myopathy of the adductors than of the internal intercostals or diaphragm. Internal nuclear rowing was not characteristic of skeletal myopathy in mink.

In the mink fed the tocopherol-deficient cod liver oil-supplemented ration, the myopathy was more extensive than that observed in the simply tocopherol-deficient mink. Many fibers, especially of the adductor muscles, had undergone calcification in the presence of many sarcolemmal nuclei. On the basis of the above suggested classification, this myopathy was considered to be early phase 3.

Although selenium supplementation of the tocopherol-deficient mink prevented the gross myopathy of tocopherol deficiency, the microscopic myopathic lesions were not prevented completely. Mink killed after having been fed the selenium-supple-

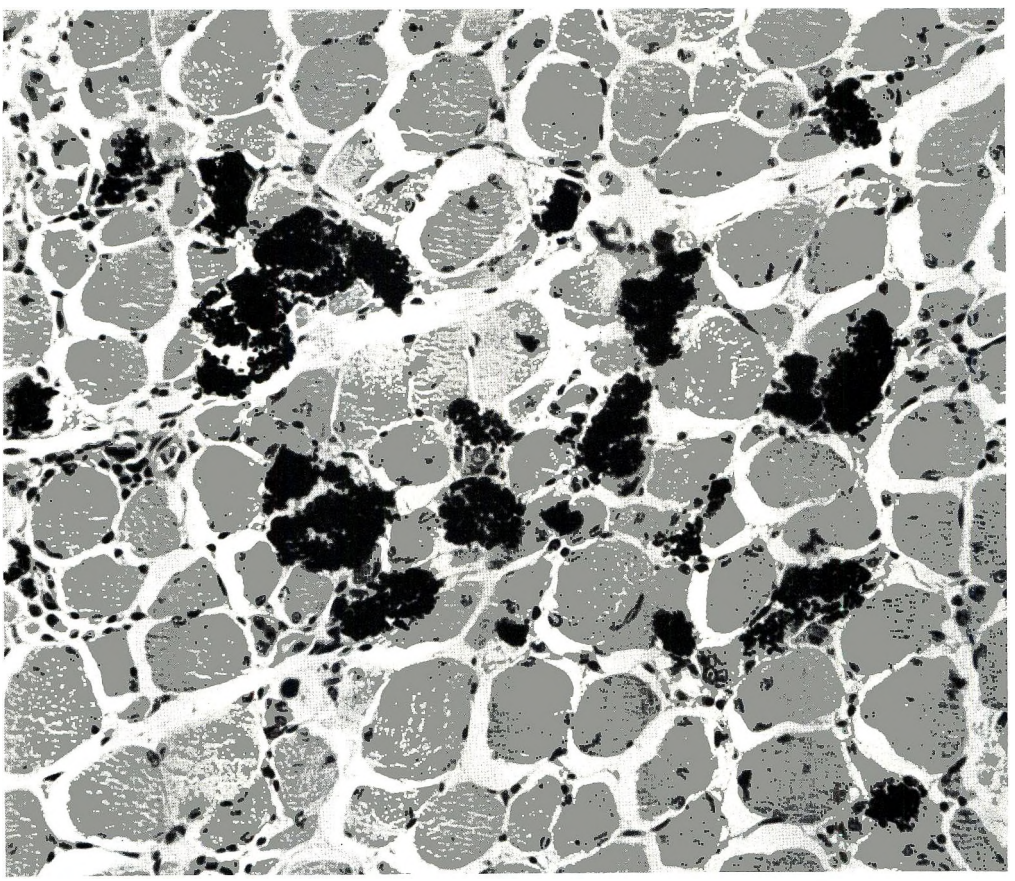

Fig. 4 Calcified foci (black) in an adductor from a tocopherol-deficient mink. Von Kossa stain × 350.

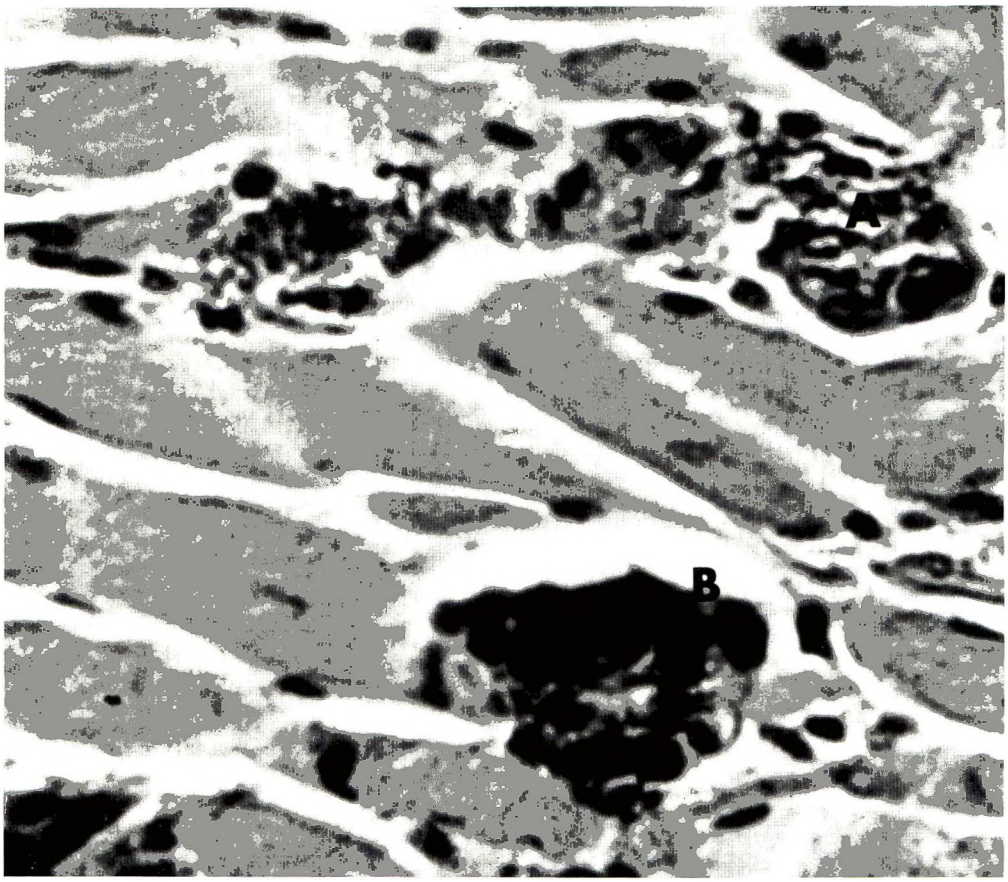

Fig. 5 Internal intercostal muscle from a tocopherol-deficient mink; (A) Early myofibrillar calcification; and (B) myophagia of calcifying muscle fiber. H & E stain × 540.

mented tocopherol-deficient ration had an early adductor myopathy characterized by an increase in the number of sarcolemmal nuclei.

Cardiac myopathy was observed grossly as irregular white patches on the endocardial surfaces from 2 tocopherol-deficient mink. Microscopically, early cardiac myopathy was distinguished by a relative increase in the number of internal nuclei which showed a tendency to row. Advanced cardiac myopathy (fig. 6) was characterized by calcified necrotic foci of variable sizes immediately adjacent to normal cardiac muscle. No intermediate stages of cardiac myopathy were observed.

Cardiac myopathy was not observed in the selenium-supplemented tocopherol-deficient mink.

Steatitis. Only the tocopherol-deficient mink that received the 8% cod liver oil ration showed gross evidence of yellow fat or steatitis. Microscopically, this condition appeared as in figure 7 with acid-fast pigment, lymphocytes and some macrophages noted in the interstitial spaces of the adipose tissue.

On the other hand, earlier stages of adipose dyscrasias were observed microscopically in the adipose tissue from tocopherol-deficient mink not receiving the highly unsaturated fatty-acid diet. When compared with the normal adipose tissue, the interstices of the adipose tissue from the tocopherol-deficient, selenium-supplemented mink appeared to contain amorphous, non-acid-fast material as in figure 8. In addition, the mink surviving chronic

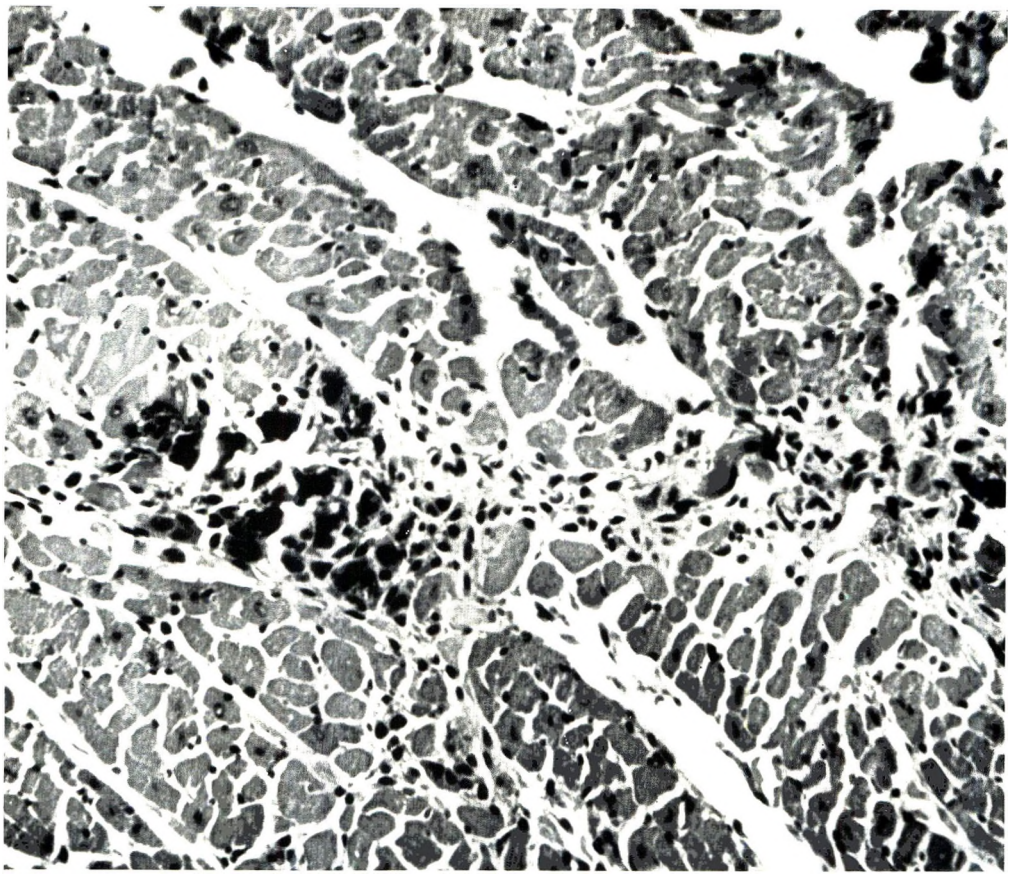

Fig. 6 Focal necrotic myocarditis with calcification in a tocopherol-deficient mink. H & E stain × 320.

tocopherol deficiency had adipose tissue whose interstices contained tiny non-acid-fast spheres which, in some ways, resembled adipocytes as in figure 9.

Hepatopathy. What appeared grossly to be a peripherolobular infiltration of fat in the liver of the tocopherol-deficient mink was confirmed upon histological examination. In addition, there was centrolobular congestion. When compared with normal portal triads, the triads of the tocopherol-deficient mink were larger than normal and edematous as in figure 10. The selenium-supplemented, tocopherol - deficient mink killed at the end of the experiments had a lymphocytic infiltration around the portal triads.

Nephropathy. Gross renal lesions were not observed in any of the tocopherol-deficient mink; however, glomerular congestion, focal capillary congestion and pyknosis in the proximal and distal convoluted tubules were observed in the tocopherol-deficient mink.

Tocopherol-deficient mink with early myopathy had hemorrhagic peritubular foci in the renal cortex and medulla. Hemosiderin was evident in the cytoplasm of the proximal and distal convoluted cells (fig. 11). A rather homogenous serosanguineous fluid was present in the intertubular spaces of the kidneys of the tocopherol-deficient selenium-supplemented mink that were killed. Bowman's capsules were often distended with a proteinaceous material which appeared to have produced some glomerular atrophy. There was evidence of hyperplasia of the juxta-glomerular appa-

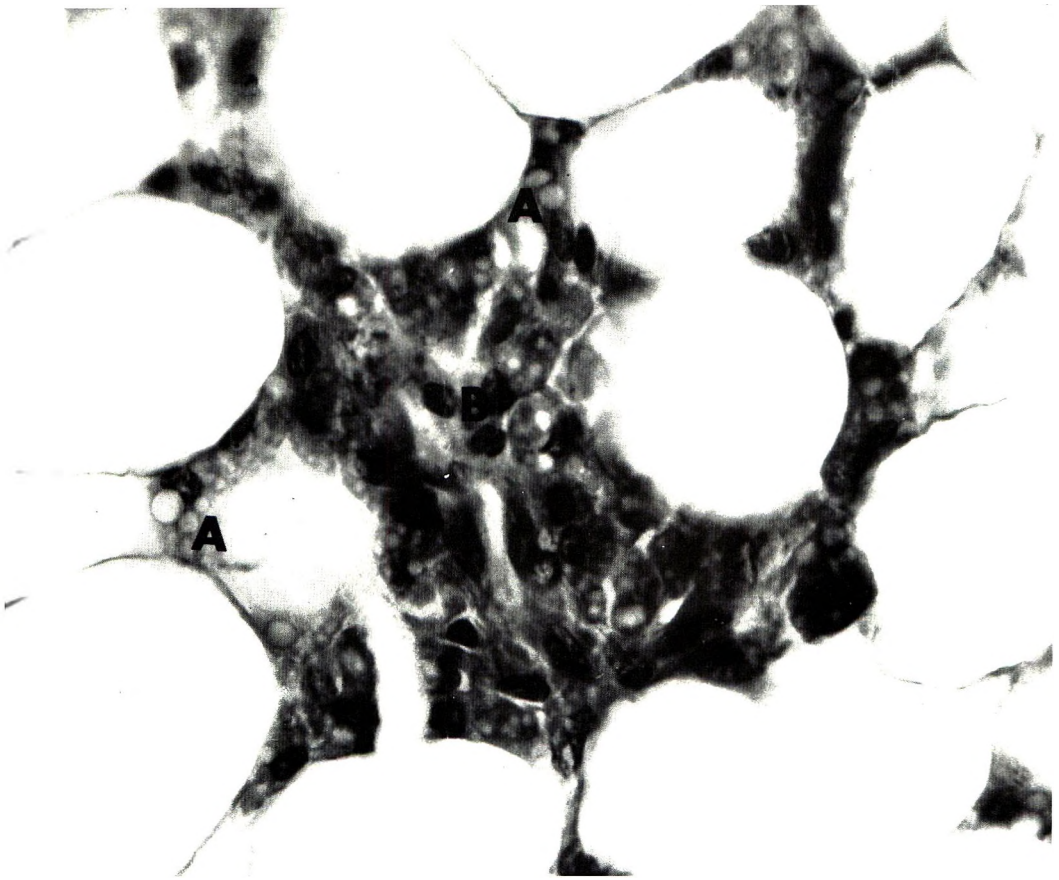

Fig. 7 Adipose tissue from tocopherol-deficient cod liver oil-supplemented mink; (A) Acid-fast pigment droplets; and (B) lymphocytes in the interstitial spaces. Ziehl-Neelsen stain × 860.

ratus cells of one mink that had an early myopathy.

In the mink with extensive myopathy, coagulation necrosis of the proximal and distal convoluted tubules was observed. Calcified foci were even present in the cytoplasm of the necrotic cells of the proximal and distal convoluted tubules of the mink fed the 8% cod liver oil ration (fig. 12).

Selenium supplementation of the tocopherol-deficient rations prevented the above renal pathology.

Adrenalopathy. Although gross changes were never observed, hemorrhagic foci were frequently observed in the adrenal cortex and medulla of the tocopherol-deficient mink. On one occasion a calcified necrotic focus was observed in the adrenal cortex. In the adrenal glands from the mink fed the 8% cod liver oil diet, lymphocytic foci were observed in the zona fasciculata.

The gross and microscopic lesions associated with tocopherol deficiency in mink were prevented by supplementing the basal semipurified type rations with 25 ppm of α-tocopherol.

DISCUSSION

Skeletal muscle and adipose tissue were the most frequently affected tissues in experimental tocopherol deficiency in mink. Myopathy was observed, however, in the presence and absence of any form of steatitis and steatitis was observed in the absence of myopathy.

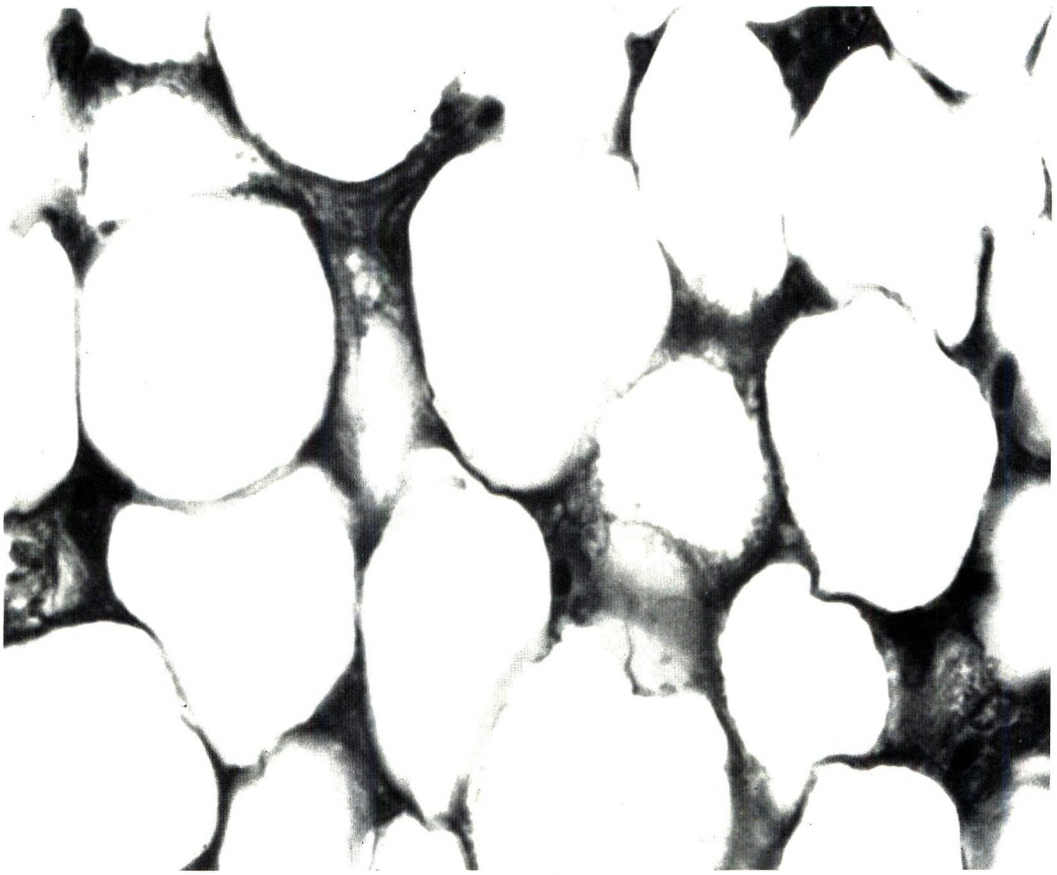

Fig. 8 Adipose tissue from tocopherol-deficient selenium-supplemented mink. Interstitial spaces contain amorphous, non-acid-fast material. H & E stain × 860.

Myopathy was often present without gross evidence of "white muscle" commonly associated with tocopherol deficiency. In such cases, the myopathy was histologically identified more readily in the adductor muscles than in the intercostal muscles. The skeletal myopathy, in general, followed the pattern of nutritional myopathy described by Adams and Denny-Brown (9). However, in mink, vacuolar degeneration appeared more characteristic of early myopathy than was emphasized by Adams and Denny-Brown. Also, the internal nuclear rowing, described by West and Mason (10) as a prominent feature of tocopherol-deficiency myopathy in hamsters was not a common feature of the same myopathy in mink.

Gross cardiac myopathy, a condition which Benson (7) reported in adult mink and called "white heart" disease, was observed only twice during these experiments. This incidence of tocopherol-deficiency cardiac myopathy is in contrast to that reported in lambs by Bacigalupo.[5] He indicated that right ventricular lesions were frequent in the experimentally produced stiff-lamb syndrome.

The overall incidence of tocopherol-deficiency myopathies in experiment 2 was less than that observed in experiment 1. This is attributed to the fact that in experiment 1, the kits were weaned onto the tocopherol-deficient rations, whereas in ex-

[5] Bacigalupo, R. A. 1952 The effects of vitamin E deficiency in the lamb. Michigan State College Ph.D. thesis.

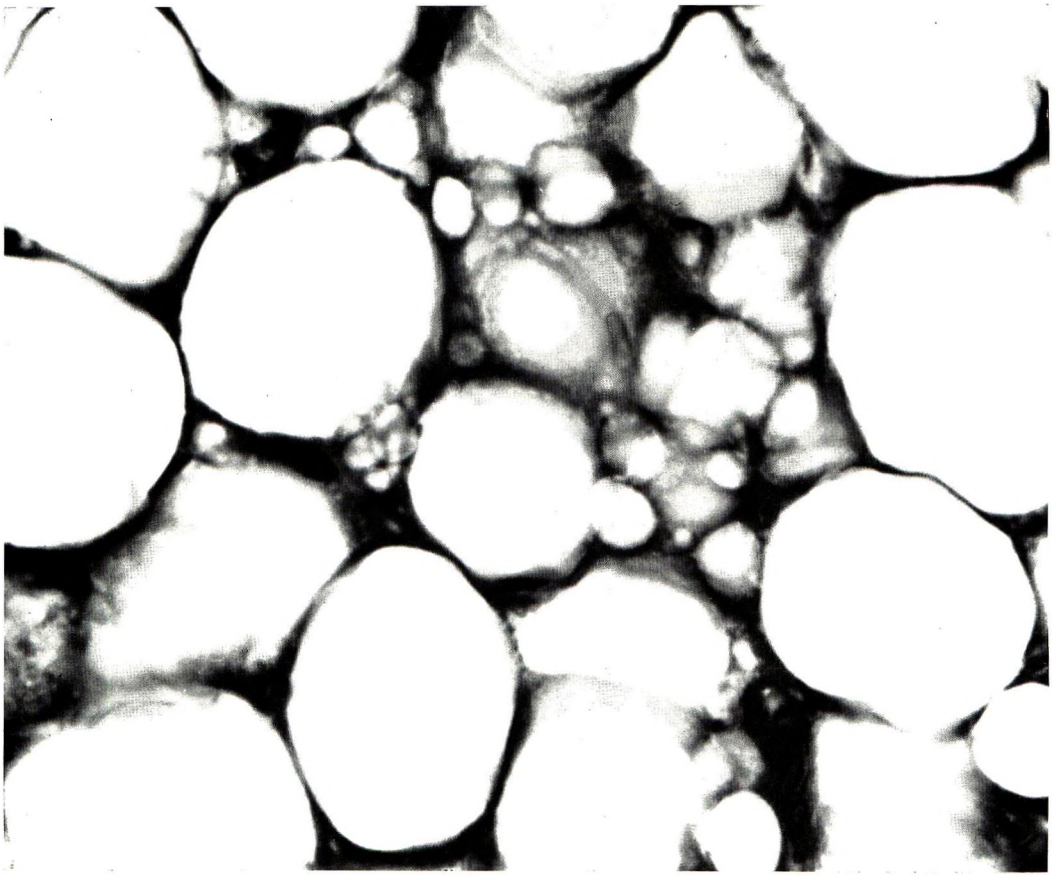

Fig. 9 Steatitis of a mink made chronically tocopherol-deficient without unsaturated fatty acid supplementation. Spheres in the interstitial spaces are not acid-fast. Ziehl-Neelsen stain × 860.

periment 2, kits were completely weaned and eating standard ranch rations before they were obtained for the experiment. Consequently, considerably more muscle development had occurred in the latter mink prior to starting to feed them the tocopherol-depletion ration than had occurred in experiment 1.

With the variety of muscle lesions observed, it should be easy to formulate the steps in the pathogenesis of tocopherol-deficiency myopathy, but the gamut of lesions is seldom observed in a single animal or a single muscle. Swollen fibers and vacuolar degeneration are often present without signs of myophagic infiltration or focal calcification. Fibers may be differentially stained or even infiltrated with myophages without accompanying evidence of vacuolar degeneration. Also, individual calcified fibers can be present without being accompanied by myopathy of the surrounding fibers or fasciculi.

When the internal rowing of skeletal muscle, described by West and Mason (10), is present, it is believed to follow vacuolar degeneration, the nuclei accumulating in the vacuoles. Vacuolar degeneration, however, does not preclude internal rowing. Internal nuclear rowing is seldom observed in tocopherol-deficient mink, whereas vacuolar degeneration is a prominent characteristic.

The sequential pathogenesis of tocopherol-deficiency myopathy in mink may be similar to the following:

1. Serum-tocopherol values below 50 μg /100 ml serum.

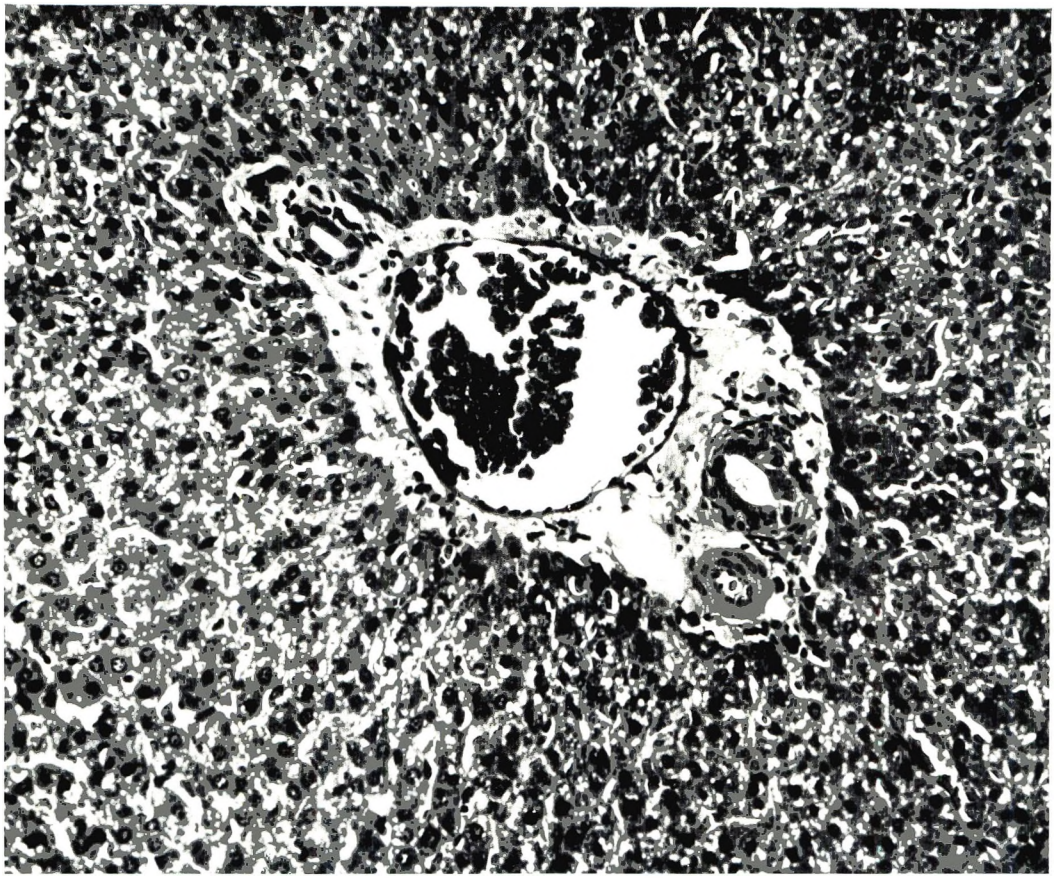

Fig. 10 Edematous portal triad from tocopherol-deficient mink. H & E stain × 230.

2. Swelling of individual fibers of the active voluntary muscles, especially the internal intercostals and the adductors.

3. Alteration of the pH of these swollen fibers indicated by differential staining characteristics.

4. Vacuolar degeneration or proliferation of sarcolemmal nuclei, or both.

5. Fragmentation and lysis of the degenerate sarcoplasmic masses.

6. Attempted regeneration of the muscle by proliferation of myoblasts in intact endomysium.

7. Phagocytosis of the necrotic myofibrils and sarcolemmal cells.

8. Calcium deposition in the phagocytized or nonphagocytized sarcoplasmic debris.

9. Restoration of function to regenerated muscle fibers.

Unfortunately, the factors which initiate this chain of events in the musculature remain more speculative than the above sequence.

Skeletal and cardiac myopathy in the absence of any form of steatitis was produced within 60 days in mink kits that were weaned onto the tocopherol-deficient and unsaturated-fatty-acid-low basal rations used in these experiments. The situation wherein myopathy was present in the absence of steatitis was considered the uncomplicated tocopherol deficiency.

Apparently 3 forms of adipose tissue dyscrasia were produced. The mildest type was characterized by the accumulation of amorphous material in the interstices of the adipose tissue from tocopherol-depleted mink supplemented with 0.1 ppm selenium.

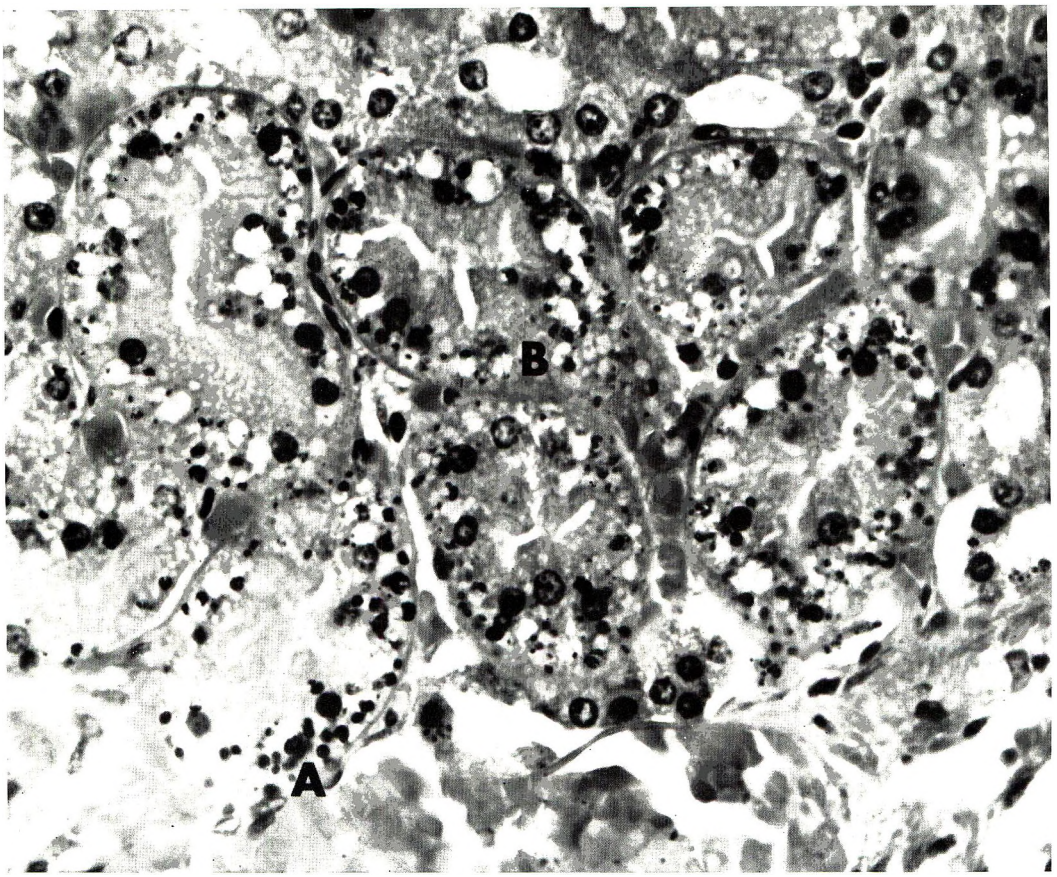

Fig. 11 Kidney of a tocopherol-deficient mink; (A) Hemosiderosis; (B) coagulation necrosis in convoluted tubules. H & E stain × 620.

The second type, characterized by microdroplet-size spheres in the interstices of the adipose tissue, was observed in the chronically tocopherol-deficient mink. The fact that this type was not observed in the selenium-supplemented group is evidence that selenium affords at least partial protection for adipose tissue during tocopherol deficiency. This is not in full agreement with the report of Edwin et al. (11) who reported that selenium supplementation of necrogenic torula yeast diets, fed to rats, gave no protection against peroxidation of the body fats.

The third type, which has been described by Hartsough and Gorham (2) and Gorham et al. (12), was characterized by the accumulation of acid-fast pigment droplets in the interstices of the adipose tissues and occurred in the mink fed the basal tocopherol-deficient ration with the isocaloric addition at 8% cod liver oil.

Although there was a neutrophilia in the mink so affected, possibly resulting from the concurrent myopathy, the neutrophilic infiltration of the adipose tissue, characteristic of steatitis in cats (13) was not observed.

The hepatic, renal and adrenal hemorrhages observed indicate that a relationship may exist between capillary permeability and tocopherol deficiency. Such evidence was provided by Grant (14) who reported upon a microangiopathy associated with tocopherol deficiencies and yellow-fat disease in swine. The angiopathy was identified by the presence of periodic acid Schiff-positive material accumulating

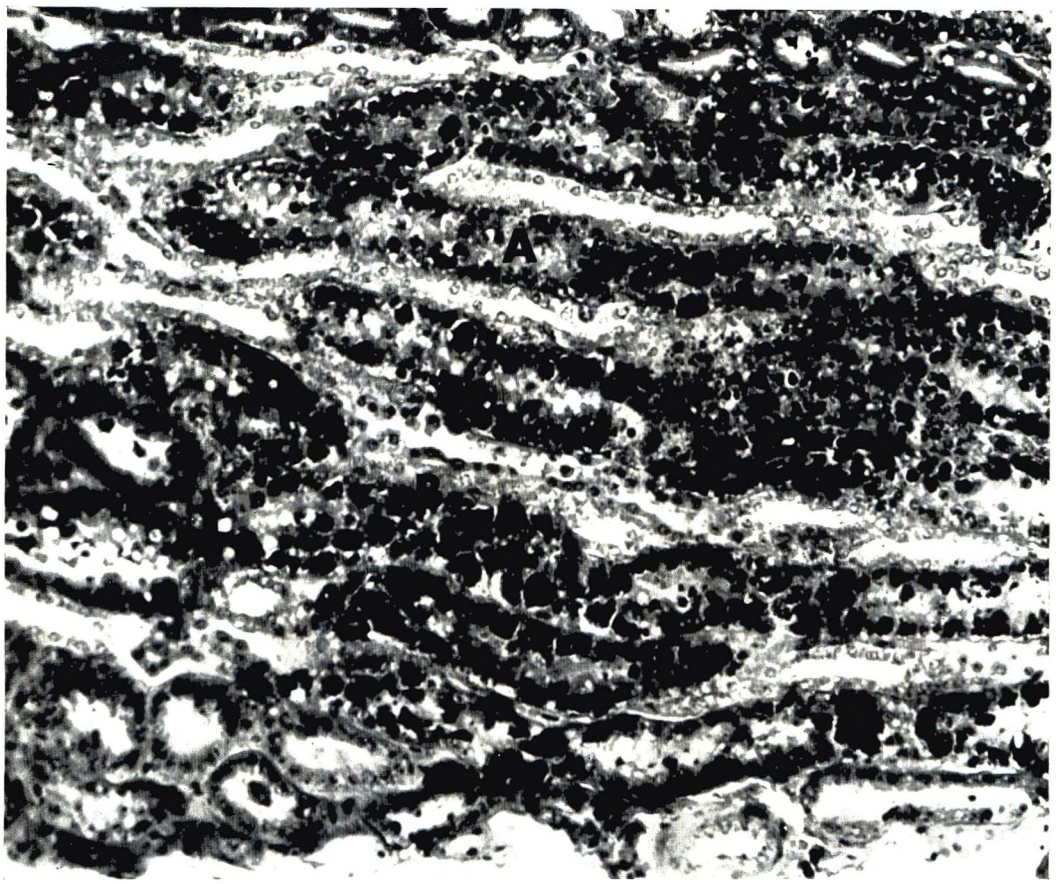

Fig. 12 Kidney of tocopherol-deficient cod liver oil supplemented mink; (A) Coagulation necrosis; and (B) calcification of the convoluted tubules in longitudinal section. H & E stain × 250.

in the subendothelial area of small arterioles and capillaries, especially in the heart. The observations of Grant in this respect were not confirmed in the mink experiments either histochemically or histopathologically.

Evidence has been presented by other researchers to indicate that the renal lesions previously attributed to tocopherol deficiency actually were the result of postmortem autolysis which is more rapid in tocopherol-deficient animals than tocopherol-supplemented animals. Figure 12, however, provides evidence that antemortem renal lesions may be present following prolonged tocopherol deficiency, especially if the tocopherol-depletion ration is relatively high in unsaturated fatty acids.

The observed adrenal pathology, however infrequent and inconsistent, supports the contention of other workers that the adrenal cortex may be the site of primary insufficiency during tocopherol deficiency. Raymondi (15) demonstrated that the action of tocopherol is comparable to that of ACTH in that tocopherol deficiency influenced the zona glomerulosa and zona fasciculata with cortical changes related to hyperemia. Hiisi-Brummer (16) presented evidence that the stress of prolonged tocopherol deficiency results in a cortical dysplasia from a hyperfunction of the adrenal cortex. Histochemical studies are required to examine further the possible insufficiency of the adrenal gland during tocopherol deficiency.

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Effect of Lysine on Calcium Metabolism in Man[1]

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ABSTRACT The effect of L-lysine monohydrochloride on calcium and phosphorus metabolism in man was studied under controlled dietary conditions. Five studies were performed in 3 patients in the metabolic research ward. The amounts of lysine added to the diet ranged from 10 to 20 g/day and the duration of the studies ranged from 12 to 30 days. These investigations revealed that the calcium balances did not improve during the administration of lysine. Radioisotope studies were performed in one patient. Only very slight improvement of Ca^{45} absorption was noted in this patient in the lysine phase, whereas the absorption of Sr^{85} remained unchanged.

The absorption of calcium has been reported to be considerably lower in mature than in growing animals (1–4). Similarly, studies carried out in man indicate differences in calcium absorption in persons of the younger and older age group.[2,3] The change in absorption with age may be partly due to a decreased need for calcium in the mature or aging organism. Increase in absorption of calcium from the intestinal tract may be desirable in certain pathologic states of calcium loss and may possibly be of benefit in persons of the older age group.

Little is known on the transfer of calcium across the intestinal mucosa and very few substances are known to increase the passage of calcium from the intestine into the blood stream. In recent years, certain carbohydrates and amino acids have been shown to improve the intestinal absorption of calcium in experimental animals (5–9). The use of lactose in man did not improve the calcium balances and has led only to a very slight increase of absorption of Ca^{45} (10).

The effectiveness of lysine on calcium absorption was investigated under controlled dietary conditions in persons of the older age group who had a relatively low absorption of ingested calcium. The results obtained are reported in this communication.

EXPERIMENTAL

The effect of L-lysine monohydrochloride on calcium, phosphorus and nitrogen metabolism was investigated in 5 studies performed in 3 patients. The studies were performed under strictly controlled metabolic conditions according to methods by Bauer and Aub (11). The age, sex, diagnoses of the patients, the amount of lysine given and the duration of the studies are listed in table 1. All patients were fully ambulatory. The physical examination did not reveal any abnormalities. Roentgenograms of the skeleton revealed the presence of osteoporosis in patients 1 and 2 and some kyphoscoliosis in patient 3. The serum calcium levels of patients 1 and 2 were normal; those of patient 3 ranged from 7.5 to 8.7 mg/100 ml. The serum phosphorus and alkaline phosphatase of the 3 patients were normal. Complete blood counts, urinalysis, fasting

TABLE 1
List of patients studied

Patient	Age and sex	Diagnosis	Lysine	
			g/days[1]	*days*
1	77 F	Osteoporosis	10	22
			10	24
2	67 F	Osteoporosis	20	12
			10	12
3	50 M	Hypopara-thyroidism	20	30

[1] Given in 3 divided doses together with calcium supplements at meal time.

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[1] Supported in part by U. S. Public Health grant A-5572 and in part by the National Society for Crippled Children & Adults, Easter Seal Research Foundation.
[2] Li, M., H. Spencer and J. Samachson 1960 Effect of high calcium intake on calcium metabolism in man. Federation Proc., *19*: 251 (abstract).
[3] Spencer, H., J. Menczel and J. Samachson. The absorption of calcium in patients with osteoporosis, in preparation.

blood sugar, liver and renal function tests were normal.

The diet was a constant, analyzed, low calcium diet which contained an average of 1850 kcal, 264 g carbohydrate, 60 g fat, 67 g protein, 152 mg calcium and 707 mg phosphorus/day. The composition of the diet is listed in table 2. The calcium intake was increased to a level of approximately 1 g calcium/day by supplementing the low calcium diet with calcium gluconate tablets. All other constituents of the diet remained unchanged. The daily fluid intake was kept constant. The control studies ranged from 12 to 36 days, the experimental studies from 12 to 30 days. In the latter phase, 10 to 20 g L-lysine monohydrochloride[4] were given orally/day in divided doses with meals, together with one-third of the daily oral calcium supplement (table 1). The body weight, fluid intake and output, and the urinary excretions of creatinine, calcium and phosphorus were determined daily.

Metabolic balance studies of calcium, phosphorus and nitrogen were performed for each patient on aliquots of 6-day pools of urine and stool collections and on aliquots of the diet. Analyses of urinary and fecal nitrogen were performed by the Kjeldahl method, phosphorus by the method of Fiske and SubbaRow (12), and urinary calcium by the method of Shohl and Pedley (13). Stool calcium and phosphorus were determined on ashed aliquots of 6-day metabolic pools.

Radioactive tracer studies were carried out in the control and experimental phase in patient 1. A tracer of Sr^{85} and Ca^{45} (0.2 μc Ca^{45} and 0.4 μc Sr^{85}/kg) was given as a single dose by the oral route with breakfast on the first day of the control and experimental studies. Plasma levels of the radioisotopes were determined at 1, 4, 8 and 24 hours after the ingestion of the dose. After the first day of the tracer study, the plasma levels were determined daily for 6 days and 3 times per week thereafter for as long as significant counts could be obtained with the equipment available. The Sr^{85} and Ca^{45} analyses of urine were determined daily on aliquots of 24-hour urine collections. Each stool specimen was radioassayed separately for Ca^{45} and Sr^{85} and analyzed for calcium. The duration of the radioisotope study was 22 days in the control phase and 14 days in the experimental phase.

The administration of tracer doses of Sr^{85} and Ca^{45} and the analyses of both radioisotopes in plasma, urine and stool have been previously described (14, 15). The Sr^{85} was counted in a well-type scintillation counter, Ca^{45} in a Q-gas flow counter after precipitation with calcium oxalate as the carrier onto special planchets.

RESULTS

Table 3 shows the metabolic balance data of calcium, phosphorus and nitrogen of the 3 patients studied. In patient 1, a slight increase of urinary calcium excretion was noted in 2 separate studies during which 10 g lysine were given daily for 22

[4] Kindly supplied by the Chas. Pfizer Pharmaceutical Company, New York, New York.

TABLE 2

Composition of metabolic low calcium diet

Carbohydrate		Protein		Fat	
	g/day		*g/day*		*g/day*
200 mg Fruit juice	24	100 g Bread	15	50 g Butterfat	41
20 g Rice[1]	16	20 g Spaghetti[1]		20 g Cream	2
100 g Bread	52	20 g Rice[1]		175 g Meat	24
70 g Jelly	46	175 g Meat (beef and turkey)	40		
20 g Spaghetti[1]	15	100 g Potatoes[2]	5		
100 g Potatoes[2]	19	200 g Canned vegetables			
200 g Canned vegetables	18				
225 g Sweetened canned fruit	44				
28 g Sugar	28				
Total	264		60		67

[1] Weight of raw food.
[2] Weight of cooked food.

TABLE 3

Effect of lysine on mineral and protein metabolism in man

Study		Calcium				Phosphorus				Nitrogen balance
		Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	
	days	*mg/day*		*mg/day*		*mg/day*		*mg/day*		*mg/day*
					Patient 1					
Control	36	1468	106	1171	+191	516	192	280	+ 44	+ 971
Lysine, 10 g/day	22	1485	152	1250	+ 83	541	240	279	+ 22	+1117
Control	12	1478	100	1344	+ 34	527	183	334	+ 10	+1329
Lysine, 10 g/day	24	1488	138	1255	+ 95	543	243	270	+ 30	+1582
					Patient 2					
Control	18	1504	305	1110	+ 89	665	428	238	− 1	+ 844
Lysine, 20 g/day	12	1495	297	1023	+175	557	350	233	− 26	+ 159
Lysine, 20 g/day	12	1492	318	999	+175	576	267	188	+121	+ 121
					Patient 3					
Control	30	1496	76	1331	+ 89	677	311	290	+ 76	+1146
Lysine, 20 g/day	30	1494	83	1267	+144	626	312	271	+ 43	+3781
Control	14	1489	103	1177	+209	613	300	234	+ 79	+1882

and 24 days, respectively. In the lysine phase the fecal calcium excretion was intermediate to that in the 2 control studies. The calcium balances of this patient did not follow any particular trend and became slightly less positive during the first study, whereas there was slight improvement of the calcium retention in the second lysine study. In patients 2 and 3 the urinary calcium excretion remained in the same range during the administration of lysine as in the control study. In patient 2 the fecal calcium excretion was somewhat lower in the 2 lysine studies than in the corresponding control studies. In patient 3 the fecal calcium excretion was intermediate during lysine intake as compared to the excretions in the 2 control studies. However, in general, these differences were small and the values were similar in the control and lysine studies. The calcium balances of patients 2 and 3 were slightly more positive during the administration of lysine than in the control study. However, the calcium balance of patient 3 was more positive following the discontinuation of lysine than during its administration.

There was no consistent change in urinary phosphorus excretion in the lysine phase (table 3). An increase of urinary phosphorus excretion of approximately 50 mg/day was noted in patient 1, whereas a decrease of the phosphaturia occurred in patient 2 and there was no change in patient 3. Also, there was no consistent effect on the excretion of fecal phosphorus and the phosphorus balances were approximately in the same range in the control and lysine studies. The changes in nitrogen balance did not show any definite trend. There was slight improvement of the nitrogen balance in patient 1, a decrease in patient 2 and a marked improvement in patient 3.

Figure 1 shows the changes in urinary calcium excretion in patient 1 in 2 lysine studies. In each phase of lysine administration there was a distinct increase in the urinary calcium excretion. This excretion returned promptly to base line levels after the discontinuation of lysine.

Table 4 shows the results of Ca^{45} and Sr^{85} studies carried out in patient 1. In the control study the urinary excretion of Ca^{45} was considerably lower than that of Sr^{85} on the day of the oral administration of the tracers. In the lysine phase there was no change in these excretions on the corresponding day. The 14-day cumulative urinary Sr^{85} excretions were similar in the control and lysine studies. However, the cumulative urinary Ca^{45} excretion was slightly higher during lysine intake than in the control study corresponding to the higher urinary calcium excretion in this phase. The fecal Sr^{85} excretion was of similar magnitude in the control and experimental phase, whereas the fecal Ca^{45}

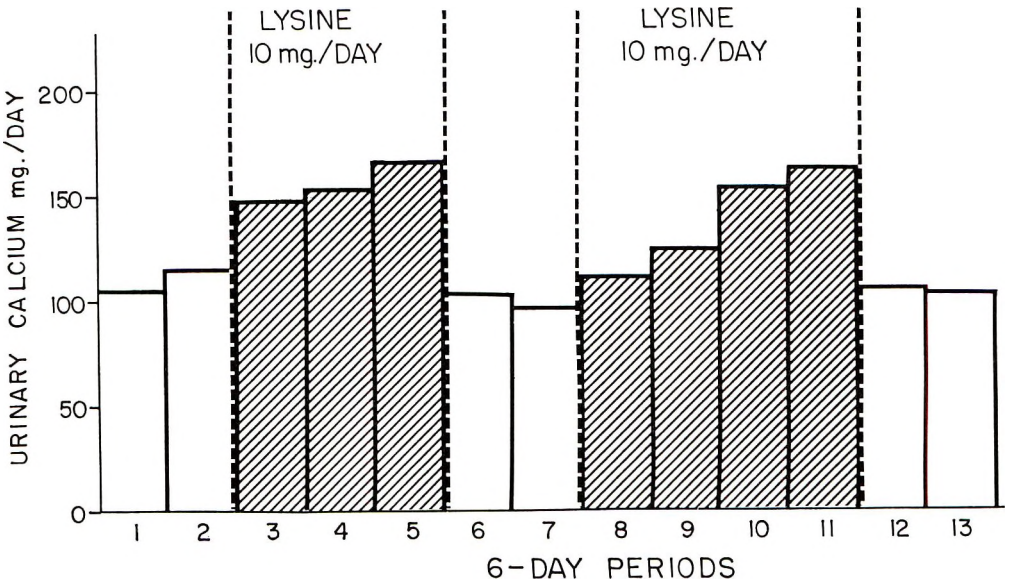

Fig. 1 Urinary calcium excretion during the administration of lysine (patient 1); each bar represents the average excretion for 6 days.

TABLE 4

Urinary and fecal Sr^{85} and Ca^{45} excretion before and during lysine administration (14-day cumulative excretions)[1]

Study	Urinary excretion			Stool	
	Sr^{85}	Ca^{45}	Calcium	Sr^{85}	Ca^{45}
	% dose	*% dose*	*mg/day*	*% dose*	*% dose*
Control	6.3	3.3	106[2]	93.8	93.8
Lysine[3]	6.5	4.7	152[2]	91.9	83.2

[1] Sr^{85} and Ca^{45} were given simultaneously as a single tracer dose by the oral route on the first day of each study. The excretions are cumulative for 14 days.
[2] Average of 14 days.
[3] 10 g lysine given/day in divided doses.

excretion was somewhat lower during the administration of lysine.

Figure 2 shows the Sr^{85} and Ca^{45} plasma levels of patient 1 following the oral administration of both radioisotopes. In the control study, the plasma level of Ca^{45} was considerably higher than of Sr^{85}. During the administration of lysine, this difference was accentuated as the Ca^{45} plasma level was somewhat higher than in the control study while the plasma level of Sr^{85} was slightly lower.

DISCUSSION

The calcium balances improved only very slightly during lysine supplementation in 4 of the 5 studies, the increase ranging from 50 to 85 mg/day. However, this effect could not be reproduced consistently in the same person. For instance, the calcium balance improved by 60 mg/day in patient 1 in one study, and decreased by 108 mg in another. The slight improvement in calcium balance was mainly due to a slight decrease in fecal calcium excretion. However, these minor changes are difficult to interpret since variations in average stool calcium excretion of approximately 100 mg/day frequently occur with a high calcium intake. A change of this order of magnitude may be due either to improvement in absorption or to physiological variations. Fluctuations in fecal calcium excretion in

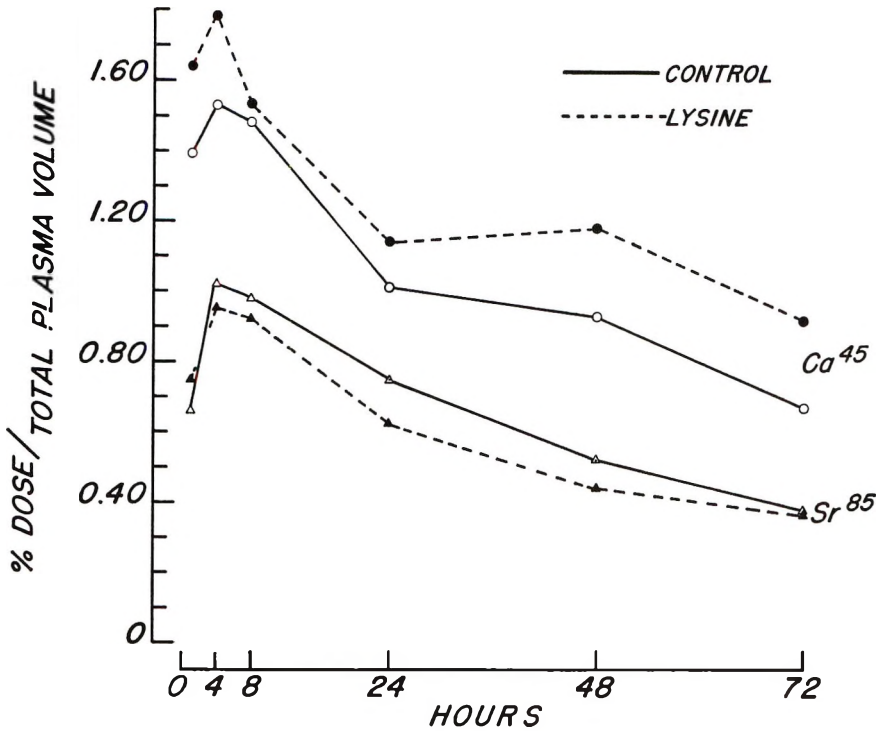

Fig. 2 Effect of lysine on Sr^{85} and Ca^{45} plasma levels in man; a single dose of Sr^{85} and Ca^{45} was given orally on the first day of the control and lysine studies.

different study periods occur commonly because of the inaccuracy in the separation of stool collections in the different study periods, and delay or irregularity in fecal passage through the gastrointestinal tract, especially in persons of the older age group. The radioisotope data indicate a slight increase in Ca^{45} absorption as judged by the higher plasma levels of Ca^{45} and the lower excretion of Ca^{45} in stool. However, these changes are within the range of physiological variation. In studies repeatedly performed with Ca^{47} in this laboratory in the same person on a constant dietary intake similar variations were noted.[5] In addition, the absorption of Sr^{85} was not increased in the studies performed during lysine supplementation.

The increase in urinary calcium excretion in the 2 lysine studies in patient 1 may be either due to some improvement in calcium absorption, or may also be ascribed to the hydrochloride portion of the molecule of lysine monohydrochloride. Ten grams of lysine monohydrochloride contain 2 g hydrochloric acid. Although this quantity is not sufficient to produce clinical symptoms of acidosis, it requires buffering action, part of which is probably supplied by the release of calcium from bone secondary to dissolution during acidosis. Studies in which ammonium chloride was aministered orally in amounts of 9 g/day (equivalent to 6.1 g HCl/day) have shown that the resistance to the action of the chloride in producing acidosis varied in different patients (16, 17). However, the urinary calcium excretion increased markedly in most cases during ammonium chloride administration.

Wasserman et al. (5) showed that a lysine-to-calcium molar ratio between 1 and 2 was needed to improve calcium and strontium absorption in rats. In the present study performed in man, the ratio, with a daily intake of 10 g L-lysine monohydrochloride and 1500 mg calcium, was

[5] Spencer, H., and J. Samachson. Variability of Ca^{47} absorption in man during a constant dietary intake, in preparation.

54.7/37.5 or 1.46, in the recommended range. With an intake of 20 g lysine/day the ratio was even higher than that recommended. Under these conditions, failure to achieve improved calcium absorption may be due to other factors. Wasserman et al. (5, 6) reported that lysine was effective when both lysine and the doses of the radioisotopes were given simultaneously in aqueous solution to fasted rats. In subsequent studies, Raven et al. (18) reported that lysine added to a basal diet did not significantly increase the retention of stable calcium, Ca^{45} or Sr^{85}, nor did it significantly increase the absorption of Sr^{85} with a low calcium diet.

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Effect of Dietary Fluoride upon the Magnesium Calcinosis Syndrome[1]

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ABSTRACT The effect of 200 ppm of dietary fluoride on magnesium calcinosis was studied in rats and dogs. Dogs fed diets containing 30 ppm Mg showed magnesium deficiency symptoms and mineralization of soft tissues. Addition of 200 ppm of fluoride to the diet prevented the development of gross aortic lesions and calcification of soft tissues but did not prevent the occurrence of other symptoms. The protective effect of fluoride did not appear to be mediated by the change in the level of serum Ca, Mg or P. In rats, dietary fluoride at 200 ppm had no protective effect on soft tissue calcification when they were fed 30 ppm Mg. Serum Mg of rats fed low Mg diet appeared to be decreased by fluoride feeding.

Magnesium status of laboratory animals has been shown to be influenced by many factors and interrelationships (1–5). Bunce et al. (6) reported that the addition of fluoride reduced mineralization of the aorta in magnesium deficient dogs and the animals showed 50 to 75% growth reduction as compared with littermates fed magnesium supplemented ration.

The experiments reported here were undertaken to investigate further the effect of dietary fluoride upon mineral deposition in soft tissues of rats and dogs fed low magnesium diets.

EXPERIMENTAL

The composition of the basal diet used in these studies was the same as diet 58-M (6) in which the mineral and vitamin supplements were modified.[2] It contained 30 ppm of magnesium and 0.5 ppm of fluoride.

One litter of weanling pups of mixed breeding and 3 litters of weanling beagles from our own kennel were divided into 4 groups of 4 to 6 pups each. They were fed test diets for 7 weeks. Precautions were taken to protect the animals from distemper, infectious hepatitis and leptospira canicola infections and from external and internal parasites.

Male weanling rats of Sprague-Dawley strain were divided into 4 groups of 8 each and subjected for 6 weeks to the same dietary treatments as outlined above for the weanling dogs.

The basal diet was fed to lot 1. Sodium fluoride was added to the diet of lot 2 at the level of 200 ppm F. Animals in lot 3 served as controls and were fed the basal diet with an additional 150 ppm magnesium in the form of anhydrous magnesium sulfate. The magnesium content of 180 ppm in this type of diet was considered marginally adequate for weanling dogs and rats (6,7). Animals in lot 4 were fed the control diet (180 ppm Mg) supplemented with 200 ppm fluoride as sodium fluoride. Food and distilled water were given ad libitum. Body weights and blood samples were obtained weekly. Blood serum was analyzed for calcium, magnesium and inorganic phosphorus. At the termination of the experiment, aortas, hearts and kidneys were analyzed for calcium, magnesium and phosphorus. Ash, magnesium and fluoride content of femurs were determined. In the case of rats, weekly blood analyses were not possible. Blood samples, kidneys and femurs were analyzed at the end of the experiment.

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[1] We are indebted to Wilson and Company, Chicago, for partial support of this project.

[2] Composition of low magnesium basal ration: (in per cent) Casein, extracted with hot ethanol, 21.0; sucrose, 66.0; lard, 8.0; salts, 4.9; and vitamins, 0.1.

The salt mixtures supplied per 100 g ration: (in grams) KCl, 1.14; NaCl, 1.00; $CaCO_3$, 1.50; Na_2HPO_4, anhydrous, 1.15; and (in milligrams) $Fe_2(SO_4)_3$, 36.0; $CuSO_4 \cdot 5H_2O$, 2.75; $MnSO_4 \cdot 1H_2O$, 1.54; $ZnCl_2$ (dry), 2.08; KI, 0.13; and $CoCl_2 \cdot 6H_2O$, 0.88.

The vitamin mixture supplied, per 100 g ration: (in milligrams) thiamine·HCl, 0.50; riboflavin, 0.30; nicotinamide, 0.90; Ca pantothenate, 0.20; pyridoxal·HCl, 0.088; folic acid, 0.030; *d*-biotin, 0.010; also vitamin B_{12}, 4.0 μg; vitamin A, 400 IU; vitamin D, 80 IU; *dl*-α-tocopherol, 5.0 mg; and choline chloride, 0.123 g.

Analytical methods. Soft tissues were dried at 105° and ashed at 600°. Since the aortic valve area was prone to calcification, they were dissected free of heart muscle including approximately 3 mm of aorta. Ashed samples were dissolved in de-ionized water with minimal amount of hydrochloric acid. The ether-extracted dried bones were ashed in the same manner. An atomic absorption spectrophotometer[3] was used in the determination of calcium and magnesium. Serum inorganic phosphorus and total phosphorus of the ashed samples were measured by the Fiske-SubbaRow method as described by Hawk et al. (8).

RESULTS AND DISCUSSION

Characteristic magnesium deficiency symptoms such as relaxation of phalanges, muscular weakness, tetany and ataxia were observed in dogs fed low magnesium diets. Addition of 200 ppm fluoride to such a diet (lot 2) did not alter the symptoms exhibited; however, the onset of tetanic seizures was earlier (about one week) than in those in the nonfluoridated animals. Food intake and growth rate of dogs fed 200 ppm of fluoride (lot 2 and 4) during the 7 weeks period were slightly less than those fed low fluoride diets of the same magnesium content. The level of magnesium was the main factor affecting growth in this experiment as shown in figure 1.

Pups fed a low magnesium diet without added fluoride developed mineralized lesions in the luminal wall of the aorta. Calcium content of aorta, heart and kidney were markedly increased. One dog in this lot showed calcification of the right ventricle wall. The addition of 200 ppm fluoride to the basal diet prevented the development of the aortic lesion. The calcium content of the heart valve, heart muscle and kidney was dramatically reduced by supplementary F^- at the rate of 200 ppm (table 1). The F^- reduced by 73% the calcium content observed in the kidney. Fluoride at the level used did not change the mineral composition of these organs when the magnesium content of the diet was 180 ppm. Since the growth rate and feed consumption were slightly decreased by the fluoride feeding, it was unlikely that the restriction of feed intake as observed by Bunce (6) was the major factor in the prevention of calcification of soft tissues.

The serum data failed to provide a clue to the protective action of fluoride against

[3] Perkin Elmer Corporation model 214, Norwalk, Connecticut.

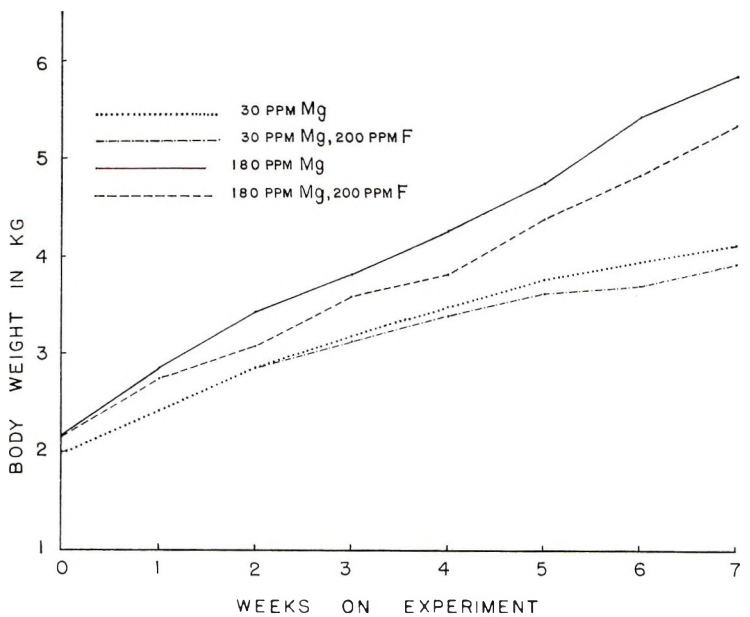

Fig. 1 Average rates of gain of dogs.

the Mg calcinosis syndrome. In both low magnesium lots serum magnesium decreased sharply to 0.6 mg/100 ml within 3 weeks and remained at this level until the termination of the experiment. The depression of serum calcium and the elevation of serum phosphate were observed, as reported by Bunce (6). No significant changes in serum composition of the elements were noticed between the fluoridated and the nonfluoridated dogs fed at the same magnesium level. Bone magnesium content, unlike the soft tissues, was greatly affected by the low magnesium diets. Magnesium content of bones from dogs fed 30 ppm Mg was about 40% of those from dogs fed 180 ppm Mg. The differences in bone magnesium levels were not affected by fluoride feeding.

Rats fed 30 ppm magnesium developed a magnesium deficiency syndrome (9). Convulsive attacks were observed in some animals fed low magnesium, with and without fluoride supplementation; however, the fluoride-fed rats showed more seizures with greater severity. Two rats fed the basal diet with fluoride failed to survive after the seizure, whereas those fed low magnesium alone continued to the termination of the study. The low magnesium basal diet retarded growth rate about 50% when compared with the positive control with 180 ppm magnesium (fig. 2). At this level of dietary magnesium animals showed no sign of deficiency. Fluoride feeding at 200 ppm did not decrease the growth of rats in this experiment.

No calcification of soft tissues was observed grossly but rats fed low magnesium diets showed a marked increase in kidney calcium. Fluoride at 200 ppm did not prevent the development of calcinosis syndrome in the rat fed 30 ppm magnesium. Bone ash was decreased by feeding low magnesium diets but was not affected by the dietary fluoride (table 2).

Average serum magnesium of rats fed the basal diet was decreased to 1 mg/100 ml, whereas fluoride feeding caused further depression to 0.76 mg/100 ml of serum (table 1). This may provide an explanation for the convulsive seizures and their severity observed in the fluoride-fed group. In contrast with the data on the dog, serum calcium of rats in the low magnesium groups was elevated and the serum phos-

TABLE 1

Analytical data, average by lot (dog)

	Lot 1	Lot 2	Lot 3	Lot 4
	30 ppm Mg	30 ppm Mg + 200 ppm F	180 ppm Mg	180 ppm Mg + 200 ppm F
No. of dogs/lot	6	4	4	5
Ash,%				
Aorta	3.23	2.21	2.05	2.02
Valve	3.44	2.52	2.33	2.40
Heart	4.79	4.44	4.62	4.99
Kidney	5.59	5.41	5.33	5.22
Bone	47.6	50.6	50.0	51.8
Calcium, mg/100 g dried tissue				
Aorta	256.7	22.1	25.5	25.1
Valve	164.3	20.1	15.6	18.2
Heart	5.0	2.8	3.1	4.1
Kidney	44.7	11.9	9.1	14.5
Magnesium, mg/100 g dried tissue				
Aorta	31.9	34.4	33.8	35.1
Valve	28.7	30.6	27.9	31.5
Heart	94	100	105	116
Kidney	65.7	72.2	63.4	69.3
Bone ash	281	317	700	700
Fluoride, ppm of ash				
Bone	98	6741	99	6634

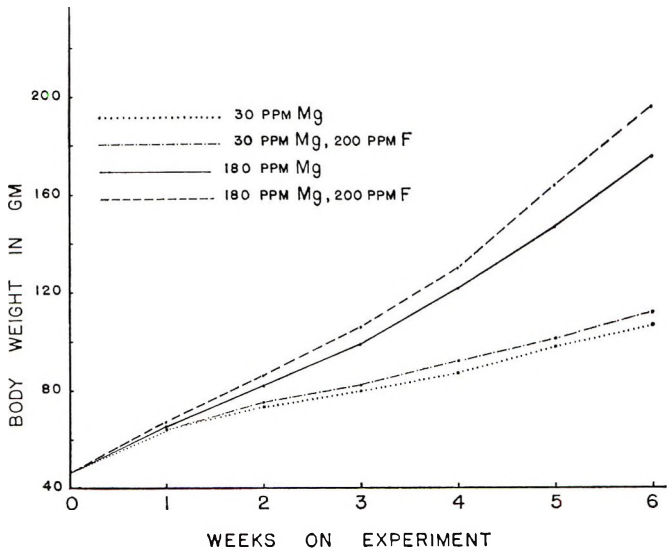

Fig. 2 Average rates of gain of rats.

TABLE 2

Analytical data, average by lot (rat)[1]

	Lot 1	Lot 2	Lot 3	Lot 4
	30 ppm Mg	30 ppm Mg + 200 ppm F	180 ppm Mg	180 ppm Mg + 200 ppm F
Serum, mg/100 ml				
Calcium	12.5	12.1	11.5	11.1
Magnesium	1.05	0.76	1.63	1.71
Phosphorus	8.95	8.63	9.25	9.91
Kidney, mg/100 g dried sample				
Calcium	174.8	131.3	5.62	5.21
Magnesium	87.2	94.4	93.1	98.6
Bone				
Ash, %	58.0	56.7	61.0	61.4
Magnesium, mg/100 g ash	211	218	580	619
Fluoride, ppm of ash	94	7848	131	7844

[1] Eight rats/group.

phate was decreased. These changes in serum composition were less pronounced with the addition of fluoride.

The data presented in figure 3 suggest species difference as measured by serum calcium and phosphorus concentrations. Dogs fed the low magnesium basal diet showed a decrease in serum calcium and an increase in serum phosphorus. In contrast the analytical evidence indicated an increase in serum calcium and a decrease in serum phosphorus for rats fed low magnesium diets. There was a decrease in serum magnesium in both species when fed low magnesium diets. In addition to the differences in serum calcium and phosphorus concentrations, the difference in protective effect of fluoride feeding against mineral deposition of the kidney was also evident. The dietary fluoride at 200 ppm did not prevent mineral deposition of the soft tissue of rats but it was effective against the magnesium calcinosis in dogs. Why these differences in serum composition and the response in fluoride feeding occurred and whether they were related are not known. Retention of fluoride in bone was unexpectedly similar in both species.

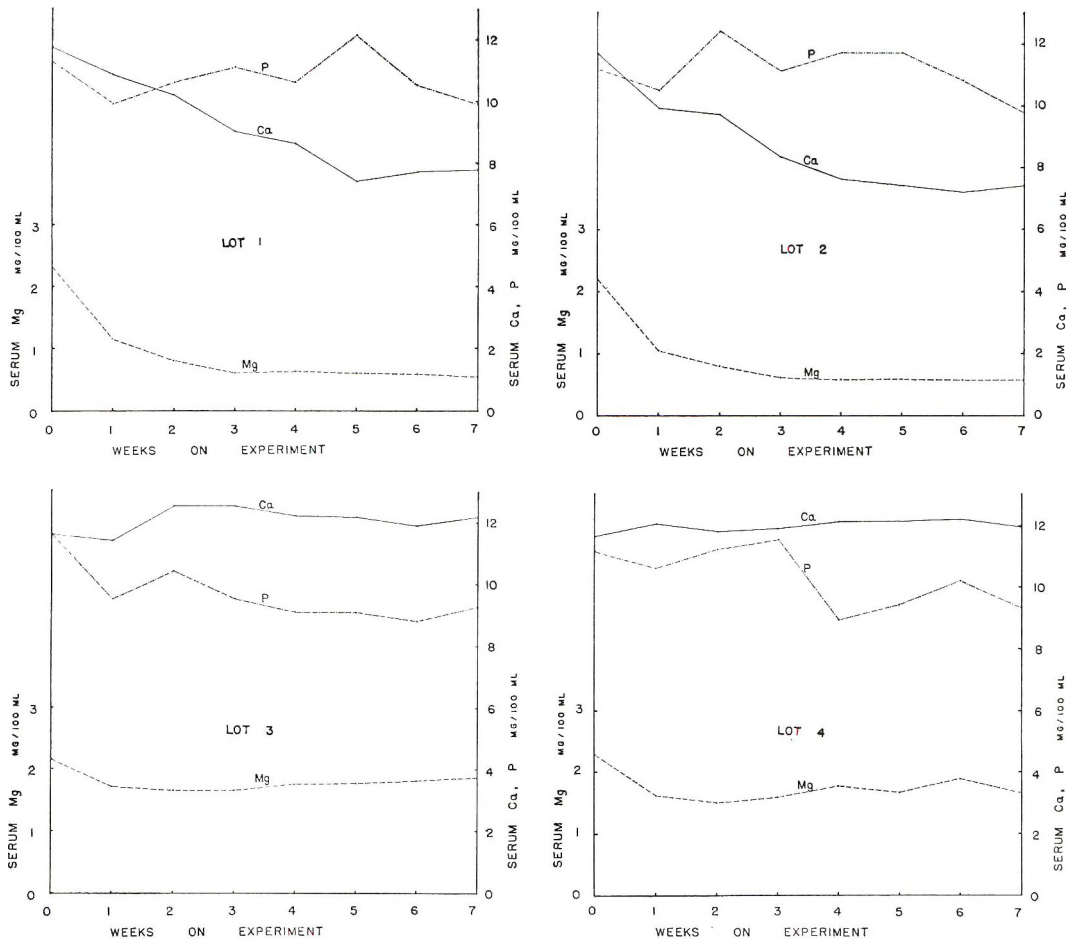

Fig. 3 Average serum Ca, P and Mg of dogs during experiment.

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Some Independent and Combined Effects of Copper, Molybdenum, and Zinc on the Placental Transfer of Zinc-65 in the Rat

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ABSTRACT The effects of increased dietary levels of different combinations of 3 elements: copper (0.01%), molybdenum (0.08%), and zinc (0.50%), were studied in the pregnant rat. Data are presented which suggest that dietary copper and zinc induce a mechanism of competition within the fetus and placental structures (amnion, chorion, yolk sac, allantois, and decidua) resulting in a lower uptake of radiozinc in these tissues. A high level of dietary molybdenum appears to be able to counteract completely the influence of an increased level of dietary copper on this uptake in the absence of increased dietary zinc, and partially overcome the influence of excess copper when it is fed in combination with a high level of zinc. Molybdenum apparently does not elicit a similar action on dietary zinc. Autoradiographic studies conducted on fetuses revealed highest activities, in order of decreasing radiozinc concentration, in: 1) calcifying bones, 2) liver, spleen and lumens of large blood vessels, and 3) kidneys. A highly significant ($P < 0.01$) increase in zinc-65 urine excretion was observed in rats fed any ration high in zinc and a significant ($P < 0.05$) increase was noted in animals fed an excess copper and molybdenum diet.

The utilization of one dietary nutrient may be profoundly influenced by the presence of another. In particular, dietary interrelationships exist between copper, molybdenum, and zinc. For example, symptoms of molybdenum toxicity in cattle respond to copper therapy (1). Conversely, supplemental molybdenum has been found to be an effective treatment of hypercuprosis in sheep (2). Kulwich et al. (3) demonstrated that excess copper may be accumulated when a high level of molybdenum is fed. Supplements of copper have been shown to partially overcome an anemia caused by high levels of dietary zinc (4–6). Molybdenum and zinc feeding has been found to reduce rat growth more than molybdenum feeding alone (7). Even molybdenum-fed rats housed in galvanized (zinc-dipped) cages have been reported to suffer more from molybdenum toxicity than those housed in stainless steel cages (8).

The placental transfer of zinc-65 under "normal" dietary conditions has been studied in the dog (9), the rat (10), and the rabbit (11). However, information concerning the placental transfer of this isotope under conditions of increased dietary levels of mineral nutrients is lacking. The metabolism of zinc has assumed greater importance since the advent of the nuclear age. Since the start of atomic weapons testing, zinc-65 has been reported in farm products (12), molluscs (13, 14), fish (15), cattle (16), and in the Marshall Island natives (17), as well as in cyclotron workers (18). Too, radiozinc has been found to be second only to radiophosphorus as an ecologically troublesome isotope in the vicinity of reactor sites (19, 20).

The purpose of the present investigation was to observe how increased dietary levels of different combinations of 3 elements, copper, molybdenum, and zinc, affected first, growth, and second, the excretion and placental transfer of intraperitoneally injected radioactive zinc in rats.

MATERIALS AND METHODS

Forty female rats of the Sprague-Dawley strain[1] initially weighing 116 to 146 g were used in this study. The animals were randomly assigned to 8 experimental groups of 5 animals each. These groups were maintained with their respective ration for 5 weeks, at which time a normal, healthy

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[1] Obtained from Sprague-Dawley, Inc., Madison 5, Wisconsin.

Sprague-Dawley male rat was housed with each experimental animal for a period of 5 days for breeding. At the end of 7 weeks all animals were given intraperitoneal injections of aqueous $Zn^{65}Cl_2$, and placed in metabolism cages. Urine and feces were collected daily until the rats were killed [2] 4 days after the tracer injection. Throughout the experimental period weight gain and feed consumption for each rat were recorded daily.

The basal diet was a finely ground commercial laboratory ration[3] containing 18 ppm copper, 70 ppm zinc, and less than 1 ppm molybdenum. One or more of the following ingredients was added to the basal diet: 0.01% copper as $CuSO_4 \cdot 5H_2O$; 0.08% molybdenum as $Na_2MoO_4 \cdot 2H_2O$; and 0.50% zinc as $ZnCO_3$.

The above levels of supplementation were used to obtain the following 8 treatments: 1) basal ration (control); 2) Cu; 3) Mo; 4) Zn; 5) Cu + Mo; 6) Cu + Zn; 7) Mo + Zn; and 8) Cu + Mo + Zn. The diets were fed ad libitum for the duration of the experiment.

For the first 7 weeks the experimental animals were maintained in cages of stainless steel construction suspended individually from above, having solid walls but wire-mesh floor and front. Dimensions were 25.4 cm long, 20.3 cm wide, and 17.7 cm high. Food containers were clear glass cylinders, 8.9 cm in diameter and 10.2 cm deep, equipped with white lacquered screw-cap metal lids having a 3.2-cm hole stamped through the middle to minimize spillage of food. The metabolism cages used during the last 4 days of the study were constructed in essentially the same fashion as described above, except for smaller dimensions and special cone dividers in the excreta collection funnel to allow more complete separation of the feces and urine. Distilled, demineralized water for drinking was dispensed from glass bottles equipped with stainless steel outlet tubes.

Four animals in each group received 1 ml of an aqueous $Zn^{65}Cl_2$ solution[4] containing 5.65 μg of zinc/ml and of specific activity 884 μc/mg. One rat in each group, used for autoradiographic studies, received 1 ml of a similar solution containing 509.05 μg of zinc/ml, specific activity 884 μc/mg. The injected doses were 5 μc and 450 μc, respectively.

Chemical and radio-analysis. After ether euthanasia fetuses and placental structures (amnion, chorion, yolk sac, allantois, and decidua) were removed, counted, and weighed. These, along with daily urine and fecal collections, were measured for radioactive zinc by counting in a well-type sodium iodide scintillation counter. Nitrogen determinations on fetuses and placental structures were carried out as described by Ferrari (21).

The rations were analyzed for total zinc by the method of Vallee and Gibson (22) using an ashing procedure described by Reitz et al. (23). The diet was analyzed for copper and molybdenum as described in the AOAC (24).

Autoradiography. Three fetuses were taken from one rat of each treatment group, fixed for 48 hours in neutral buffered 10% formalin, and then embedded in paraffin by routine procedures. Tissue sections of 5-μ thickness were cut, mounted on clean glass microscope slides, and stained first with hematoxylin-eosin and then with alizarin red S and the von Kossa stains for the demonstration of sites of mineralization of bone. Autoradiographs were prepared from replicate tissue sections using Kodak AR-10 stripping film following the technique of Pelc (25). The autoradiographs were exposed for 30 to 62 days, developed 5 minutes in Kodak D-19 developer, and fixed for 5 minutes in Kodak acid fixer with hardener. They were then stained for 10 minutes with nuclear fast red, dehydrated, and mounted with Permount. One tissue section from each fetus was dipped in 1% celloidin in ether: alcohol prior to preparing the autoradiograph in order to eliminate any possible misinterpretation due to chemical exposure of the film.

RESULTS AND DISCUSSION

Growth, feed consumption, and litter size. Growth of animals in the experimental groups did not differ significantly

[2] The Principles of Laboratory Animal Care as promulgated by the National Society for Medical Research were observed.

[3] Purina Laboratory Chow, Ralston Purina Company, Davenport, Iowa.

[4] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

from those in the control group except for those animals fed diets containing both excess molybdenum and excess zinc (i.e., Mo + Zn and Cu + Mo + Zn). Both combinations were equally efficient in growth retardation (t test). Growth in these 2 groups was significantly ($P < 0.01$) less than in the control group (table 1). These observations are in agreement with those of previous studies which show that excess zinc, when present in the diet with excess molybdenum, causes more growth retardation than either excess molybdenum or zinc alone (7, 8). The observation that elevated dietary molybdenum or zinc alone causes significant growth retardation (4, 5, 7, 26) was not noted at dietary levels of added mineral nutrient used in this study.

TABLE 1

Average weight gain of animals in the various treatment groups

	Treatment	Wt gain
		g
1	Basal ration (control)	115
2	Mo	108
3	Cu	131
4	Zn	109
5	Cu + Mo	104
6	Mo + Zn	86++
7	Cu + Zn	124
8	Cu + Mo + Zn	90++

++ Indicates a significant difference at the 1% level when compared with control animals (t test).

There was no significant difference in food consumption among treatment groups during the study. Thus the suggestion that some of the toxic effects produced in animals maintained with a ration containing molybdenum is the result of decreased dietary intake (27), perhaps due to a mechanism of sensory recognition of the presence of molybdenum in the diet demonstrated by Monty and Click (28), would not apply in this study. Johnson and Miller (29) reported a reduction in the efficiency of feed utilization when rats were fed rations containing molybdenum. A mechanism such as this may account for the weight loss in the experimental animals of this study when increased dietary zinc was present to "enhance" the effect. Whether the loss in efficiency is effected through faulty digestion or absorption of nutrients or through some other derangement in metabolism is still unanswered. No significant differences in number of fetuses per litter, wet weight of the litter, or average weight per fetus were seen among the treatment groups (table 2, lines 1 and 2).

Distribution of injected zinc-65 in the fetus and placental structures. The distribution of the injected radiozinc was evaluated in terms of the percentage of injected zinc-65 retained 4 days after administration per: 1) fetus or placental structures; 2) gram of wet weight of fetus or placental structures; 3) milligram of nitrogen in the fetus or placental structures; or 4) total wet weight of litter.

The results show clearly that the percentage of radiozinc retained by either the fetus or the placental structures within any treatment group is directly proportional to its wet weight (table 2, lines 3 and 4). That is, although there is a possible variation up to 5 days in fetal age, with resultant variation in fetal weight, when either fetuses or placental structures were evaluated in terms of activity per gram or wet weight the values obtained within any treatment group are essentially identical. Therefore, when using this basis of evaluation not only can significant differences between treatments be shown in fetuses or placental structures of comparable weight and development, but the same significant differences are noted when all fetuses or placental structures of all weights within each treatment are considered as a single group and compared. Thus, the variation in percentage of injected dose observed within a given treatment group, whether evaluated in terms of activity per fetus, per placental structures, or per litter can be attributed solely to the variation in the wet weight of that tissue being compared.

There seems to be little correlation between the percentage activity and the nitrogen content (table 2, lines 5 and 6). This observation, combined with the data indicating that radiozinc activity varies with wet weight, appears to imply that the radioactive zinc is present mainly in the tissue fluids. Since the metal is assumed to be primarily associated with protein when it is physiologically active (30), the present evidence suggests that, at 4 days

TABLE 2

Percentage of retained zinc-65 present in fetuses and placental structures 96 hours after administration

	Basal ration (control)	Mo	Cu	Zn	Cu + Mo	Mo + Zn	Cu + Zn	Cu + Mo + Zn
	%	%	%	%	%	%	%	%
1 No. of fetuses/litter	10.80	12.25	9.00	10.80	11.00	10.25	13.75	12.33
2 Total wet weight of litter, g	4.03	5.17	5.49	5.60	3.58	3.29	4.01	3.64
3 % of dose/g (wet weight) of fetus	0.233	0.240	0.199^{+}	0.165^{++}	0.303^{++}	0.180^{+}	0.108^{++}	0.134^{++}
4 % of dose/g (wet weight) of placental structures	0.242	0.237	0.196^{+}	0.167^{++}	0.330^{+}	0.178^{+}	0.133^{++}	0.140^{++}
5 % of dose/mg of nitrogen in fetus	0.0263	0.0223	0.0251	0.0263	0.0283	0.0126	0.0088^{+}	0.0152
6 % of dose/mg of nitrogen in placental structures	0.0125	0.0102	0.0098	0.0089^{+}	0.0133	0.0132	0.0058^{+}	0.0073

$^{++}$ Indicates a significant difference at the 1% level when compared with control animals (*t* test).
$^{+}$ Indicates a significant difference at the 5% level when compared with control animals (*t* test).

after injection, most of the zinc-65 is not fixed at organic sites of function.

Within each treatment group the activity per gram of fetus and activity per gram of placental structures are almost identical (table 2, lines 3 and 4). This is not too unexpected when one considers the structure of the rat placenta. According to Arey (31), the placentas of rats, guinea pigs, and rabbits are classified as hemoendothelial, demonstrating the nearest approach to actual intermingling of the blood of the fetal and maternal circulations. Chorionic villi lose their layers to such a degree that, in most places, the bare endothelial lining of their vessels alone separates the fetal blood from the maternal sinuses. This near intermingling of fetal and maternal circulations may allow almost free flow of fluid and radiozinc from maternal placenta to fetus. Moreover, Terry et al. (11) have shown that the intact placentas of rabbits, remaining after the removal of the fetus, readily accumullate radiozinc, suggesting the removal of zinc from the maternal blood is not dependent on a fetal acceptor.

Zinc-65 uptake in animals fed a diet containing only increased molybdenum was not significantly different than that observed in the control animals. However, with excess zinc alone in the diet a highly significant ($P < 0.01$) retardation of zinc-65 uptake occurred and when copper alone was added to the basal ration a significant ($P < 0.05$) retardation of radiozinc uptake was noted. Excess molybdenum fed in combination with excess copper caused a significant increase ($P < 0.05$) in fetal-placental structures uptake rather than a decrease, whereas molybdenum plus zinc fed rats showed significantly ($P < 0.05$) lower zinc-65 uptake than normal, but were about equal (*t* test) to those rats fed a diet of high zinc alone. Uptake in animals fed a copper plus zinc ration was greatly retarded ($P < 0.01$). The magnitude of the effect of feeding copper and zinc in combination is more clearly observed when it is noted that the retardation of uptake in those animals was significantly greater than the uptake in animals fed a diet of added copper alone ($P < 0.01$) or zinc alone ($P < 0.05$). It has already been pointed out that the fetus-placental

structures' uptake of radiozinc in animals of these 2 latter groups is significantly lower than that of animals in the control group. Those animals maintained with a high dietary level of all 3 trace metals showed uptakes significantly lower than normal ($P < 0.01$). Although significant differences as determined by the Student's t test were not found, the percentage uptake in both fetuses and placental structures in those animals fed a copper plus molybdenum plus zinc diet fell within a range between that of animals fed a zinc or copper diet and those supplied with a copper plus zinc diet.

These data show that added dietary copper and zinc, whether fed alone or in a combination, significantly lower the fetus-placental structures' uptake of injected zinc-65. A diet high in copper plus zinc was more effective in lowering radiozinc uptake than when either was present alone in the ration. Molybdenum fed together with increased levels of zinc apparently does not alter the lowered zinc-65 uptake caused by dietary zinc. However, molybdenum, when present in the diet with excess copper, significantly mitigates the influence of the dietary copper upon zinc-65 uptake. The effect of the dietary molybdenum is so pronounced that the animals fed molybdenum plus copper had a significantly higher fetal or (placental structures) uptake of radiozinc than the control animals. It is difficult to explain why the copper plus molybdenum combination allows more tissue uptake of zinc-65 than normal. The influence of molybdenum on copper is again demonstrated in those animals maintained with a diet high in all 3 trace elements. Molybdenum appears to suppress the influence of copper enough to allow zinc-65 uptakes in this group to fall between those observed in zinc- or copper-fed animals and animals fed the powerful copper plus zinc combination.

Autoradiography. The distribution of zinc-65 within fetuses from different treatment groups was similar. Activity above background was present over the entire fetus. The highest concentration of radiozinc occurred in calcifying bones (fig. 1) in those areas where mineralization had occurred, as determined by the von Kossa and the aliziran red S stains. In bones where mineralization had not occurred radiozinc deposition was essentially absent. This was observed in vertebrae, ribs, long bones of the extremities, and membranous bones of the skull. Houmont (32) has demonstrated a similar observation. He described radiozinc deposition in osseous tissue immediately prior to its calcification. Liver, spleen, and the lumens of large blood vessels showed relatively high concentration of deposited radiozinc although the levels did not approach that of calcifying bone. Very active hematopoiesis was observed in both the liver and spleen, and numerous free erythrocytes were noted in the lumens of the large blood vessels. Scatter was too great to directly associate activity with any particular cell type. The kidney contained a slightly higher activity than the remaining organs and tissues.

Excretion. Fecal elimination of radiozinc in the animals of the experimental groups was not significantly different. The results in this study are in agreement with the well documented observation that this route of excretion is the principal means of eliminating administered radiozinc (10, 33–35).

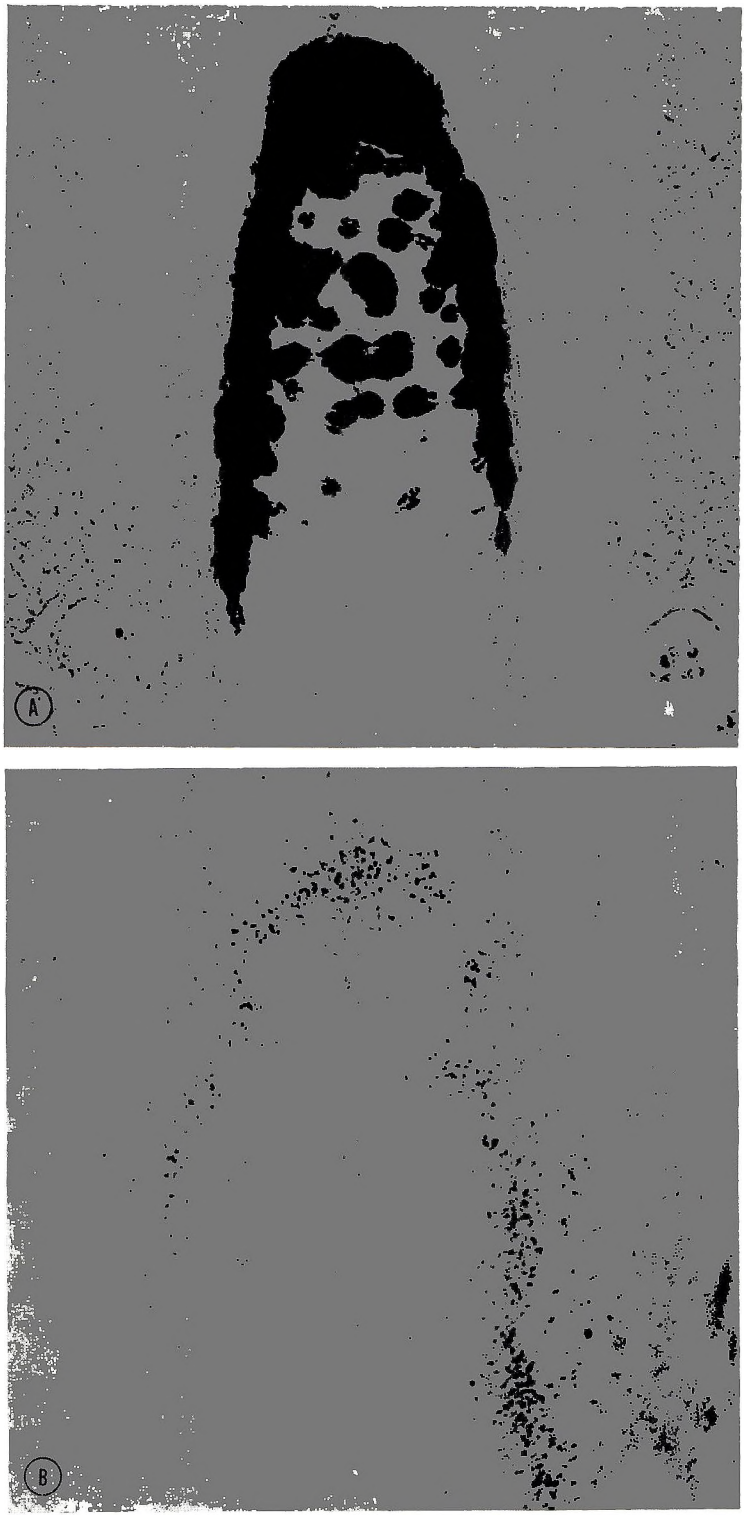
The amount of activity eliminated via the urine in 4 days is very small compared with that by fecal excretion. However, significant differences in radiozinc excretion are shown in this study (table 3). In all animals that were fed a ration containing excess zinc, the urinary zinc-65 elimination was greatly increased ($P < 0.01$). The ration containing all 3 trace minerals was more effective than the other three ($P < 0.05$). In those animals fed a copper

TABLE 3

Percentage of injected zinc-65 excreted in the urine 96 hours after administration

		%
1	Basal ration (control)	0.462
2	Mo	0.573
3	Cu	0.451
4	Zn	3.281++
5	Cu+Mo	0.730+
6	Mo+Zn	2.433++
7	Cu+Zn	4.085++
8	Cu+Mo+Zn	5.109++

++ Indicates a significant difference at the 1% level when compared with control animals (t test).
+ Indicates a significant difference at the 5% level when compared with control animals (t test).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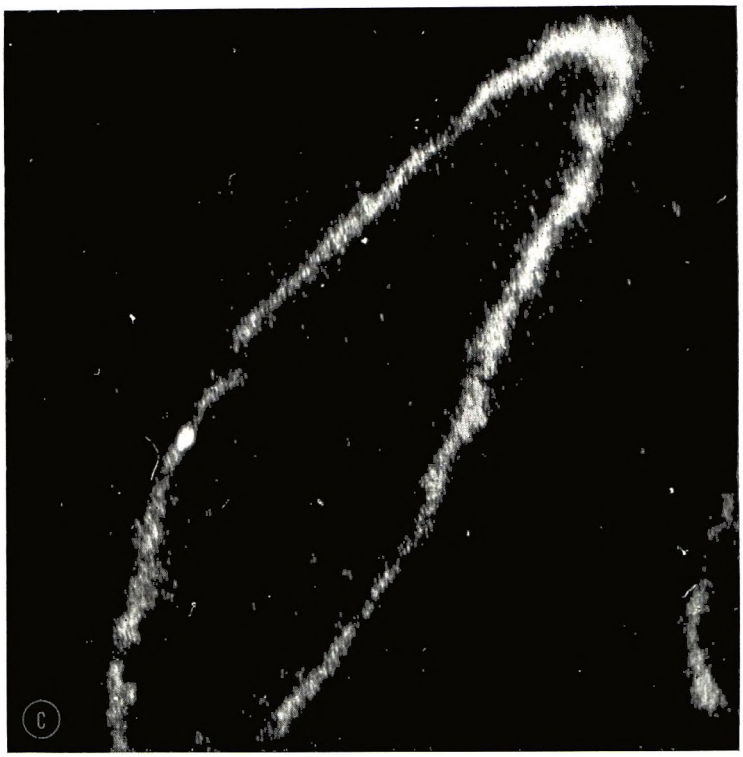

Fig. 1 Uptake of zinc-65 in calcifying bones of a normal rat fetus of the third trimester.

a. Photomicrograph of a vertebra stained with alizarin red S. The large black areas indicate sites of calcification.

b. Autoradiograph of a vertebra of a fetus in which the mother had been given 450 μc of zinc-65, 96 hours before it was killed. The black exposed silver grains indicate the sites of the isotope. Note the similarity to the sites of calcification shown in figure 1a.

c. Same as figure 1b, viewed by dark-field microscopy.

plus molybdenum diet the rate of elimination was significantly higher ($P < 0.05$). Cotzias et al. (35), using a whole-body counting technique, have shown an increased radiozinc excretion associated with high dietary zinc, but did not find significantly increased zinc-65 elimination associated with high dietary copper. A similar technique has recently been used to demonstrate decreased absorption of orally administered radiozinc in rats fed an elevated level of dietary zinc (36).

The manner in which the interactions of these trace minerals takes place is poorly understood. Magee and Matrone (37) have suggested that the interaction between zinc and copper may be caused by zinc increasing the excretion and decreasing the utilization of copper. Cox and Harris (26) stated that the antagonistic effect of zinc and copper may be a reflection of an interference on iron metabolism by zinc which is partially counteracted by the ability of copper to mobilize iron in the liver when the iron is at a very low level. Halverson et al. (38) pointed out that molybdenum may produce its interaction with copper by reducing levels of sulfide oxidase, thus leading to an accumulation of inorganic sulfide. This accumulation of sulfide presumably precipitates as highly insoluble copper sulfide. It has been pointed out, however, that if an excess production of sulfide were the specific mechanism, then other metals that form highly insoluble sulfides such as iron, manganese, zinc, and cobalt should also be members of the deficiency complex (39).

Data presented by Mills (40) indicate that the mode of metabolic disturbances caused by molybdenum-copper interaction may vary. Referring to the rat versus the ruminant, he states that the toxicity observed upon molybdenum feeding to the former is probably the result of a failure of copper utilization within the tissue. Metabolic disturbances consequent upon the feeding of molybdenum to ruminants, however, are probably caused by changes within the digestive tract which restrict copper absorption and thus are likely to be the underlying cause of the ensuing copper deficiency.

Further studies are being conducted in this laboratory, with particular emphasis upon the influence of sulfate, to define more clearly the roles of these nutrients and their interrelationships.

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Effect of Oleic and Linoleic Acids on the Absorption of Saturated Fatty Acids in the Chick[1]

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ABSTRACT Palmitic and stearic acids, which are utilized very poorly by the chick when fed singly, are absorbed to a significant degree when fed in a mixture of fatty acids such as hydrolyzed lard. Experiments were conducted with chicks to determine whether oleic and linoleic acids are specifically required for absorption of the saturated fatty acids. Increasing the amount of oleic acid (O) in relation to palmitic acid (P) resulted in a linear increase in absorption of the palmitic acid. A significant improvement was detected with an oleic to palmitic acid ratio (O:P) of 0.1:1. The absorbability of palmitic acid was increased to 80% when the O:P ratio was 0.8:1, and maximal absorption of 85 to 90% was obtained with an O:P ratio of 1.34:1. The effect of linoleic acid (L) was less dramatic. The maximal improvement in absorption of palmitic acid was 20% with the highest levels of linoleic acid. Mixtures containing a ratio of linoleic to palmitic acid (L:P) of 1:1 or more were no better than a ratio of L:P of 0.4:1. Palmitic and stearic acids when fed together tended to depress the absorption of each other. In mixtures of these 2 saturated fatty acids, high levels of oleic acid were required for 50% absorption of each of the saturated fatty acids. When linoleic acid was fed with oleic acid and a mixture of the 2 saturated fatty acids, maximal absorption of the saturated fatty acids was obtained with lower levels of both of the unsaturated fatty acids. Oleic acid appears to play a direct role in facilitating the absorption of the saturated fatty acids. It is not known whether this effect is exerted in the lumen or in the mucosal cells of the intestine.

Both stearic and palmitic acids were observed by Renner and Hill (1) to be virtually unutilized by the chick when fed singly in the diet. However, when these saturated fatty acids were fed in a mixture of fatty acids such as hydrolyzed lard or beef tallow, both Renner and Hill (2) and Young (3), using gas chromatographic analysis of the dietary and fecal fatty acids, showed that there was a significant improvement in the absorbability of stearic acid and particularly of palmitic acid.

It is generally recognized that any given fat or fatty acid mixture that contains a high proportion of saturated fatty acids is less well utilized by all species than the more unsaturated fats. Early workers attempted to correlate the degree of utilization with melting point of the fat (4–6). However, Scribante and Favarger (7) and Calloway et al. (8) showed that melting point is not a good index of digestible coefficient, but rather that the relationship of digestibility to melting point is coincidental rather than causal.

The importance of the degree of saturation upon fat utilization by chicks was reported by March and Biely (9). Later work by Renner and Hill (1,2) and Calloway et al. (8) indicated that the chain length of the saturated fatty acids and their arrangement within the triglyceride molecule are important factors in determining the extent of fat digestibility in the chick. In addition, Renner and Hill (2) and Young (3) have demonstrated that the absorbability of mixtures of fatty acids by the chick was influenced by the presence or absence of an ester linkage to a glyceride moiety.

Renner[2] showed that the absorbability of palmitic acid was improved from 5% when fed as free palmitic acid up to 35% when fed as monopalmitin and up to 40% when fed as methyl palmitate; tripalmitin was no better utilized than palmitic acid by itself.

No glycerides were present in the fatty acid mixtures used by Renner and Hill (2) and Young (3); therefore, it appears

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[2] Renner, R. 1960 Factors affecting absorbability of saturated fatty acids in the chicken. Ph.D. Thesis, Cornell University.

that the improved utilization of the stearic and palmitic acid in hydrolyzed lard and beef tallow may have been due to the presence of the unsaturated fatty acids in the free fatty acid mixtures. The experiments reported in this paper were conducted to determine the extent to which oleic and linoleic acid would influence the absorbability of the saturated fatty acids, palmitic and stearic, when these fatty acids were fed in controlled ratios of saturated to unsaturated fatty acids.

EXPERIMENTAL

To eliminate the influence of ester linkages or glycerides (2,3) on absorbability of the fatty acids, only free fatty acids were used as a source of dietary lipid. In experiments 1 and 2 hydrolyzed olive oil and hydrolyzed safflower oil (table 1, samples C and D) were used as the source of oleic acid and linoleic acid, respectively. Hydrolysis was carried out by the procedure described by Young et al. (10). Technical grades of palmitic acid and stearic acid (table 1, samples A and D) were used as sources of the saturated fatty acids.

In the third experiment, palmitic, oleic and linoleic acids of a purity of 96% or better (table 1) were used. In every case the composition of the fatty acid preparation was determined by gas liquid partition chromatography of the methyl esters of these fatty acids. These various sources of fatty acids were used to prepare specifically formulated mixtures of fatty acids in which the saturated and unsaturated fatty acids were blended at definite ratios. The exact ratio of the mixtures which the chick consumed was determined by analysis of the lipid material extracted from the experimental diets. The fatty acids were substituted on a dry-weight basis for glucose in the basal diets. Unless othewise noted, the percentage of total fatty acids substituted for glucose was kept between 20 and 23% of the diet.

The basal diets used in these experiments are shown in table 2. Diet A was used in experiments 1 and 2, and diet B in experiment 3. With the substitution of 20% soybean oil, weight for weight, for glucose, both diets provide 13.2 kcal of metabolizable energy for each gram of dietary protein. Since the chick regulates its daily food consumption to satisfy its energy requirement, it is necessary to have protein related to the energy content of the diet to insure an adequate intake of protein. Therefore, when the various fatty acid mixtures were used, this ratio was always 13.2 M.E. kcal/g of protein or less, since none of the mixtures exceeded the metabolizable energy value of soybean oil.

In experiments 1 and 2, one-day-old White Plymouth Rock cockerels were fed diet A containing 20% soybean oil for the first 14 days. The chicks were then allotted on a weight basis into equal lots of 8 chicks each. Duplicate lots of chicks were fed each experimental diet for 2 weeks.

A different procedure was used in experiment 3. More than twice the number of chicks needed were fed basal diet B

TABLE 1

Fatty acid composition of various sources of fatty acids [1]

	Sample	16:0 [2]	16:1	18:0	18:1	18:2
A	Palmitic [3]	92.0	—	8.0	—	—
B	Stearic [4]	8.4	—	91.6	—	—
C	Oleic (hydrolyzed olive oil)	13.5	2.5	3.1	71.3	9.6
D	Linoleic (hydrolyzed safflower oil)	6.3	—	1.8	14.8	77.1
E	Palmitic [5]	99.57	—	0.43	—	—
F	Oleic [5]	2.48	—	0.90	96.62	—
G	Linoleic [5]	—	—	—	—	100.0

[1] The composition of each new source of fatty acid was determined prior to use by gas-liquid partition chromtography at 185°C with a 2-meter × 4-mm inside diameter aluminum tubing packed with 16% diethylene glycol succinate supported on 60- to 80-mesh chromosorb W.
[2] The first figure represents the number of carbon atoms; the second, the number of double bonds.
[3] Neo-Fat 16, Armour Industrial Chemical Company, Chicago.
[4] Neo-Fat 18, Armour Industrial Chemical Company, Chicago.
[5] Fatty acids obtained from Applied Science Laboratories, Inc., State College, Pennsylvania.

TABLE 2

Experimental basal diets

Ingredients	Diet A	Diet B
	%	%
Ground yellow corn	14.9	—
Soybean meal (50% protein)	7.0	—
Fish meal (60% protein)	5.0	—
Dried whey	2.0	—
Corn distillers solubles	2.5	—
Dehydrated alfalfa meal	2.0	—
Isolated soybean protein [1]	25.0	35.47 [2]
Cellulose [3]	—	3.0
DL-Methionine	—	0.8
Glycine	—	0.3
Vitamin mix no. 1 [4]	1.1	—
Vitamin mix no. 2 [5]	—	1.0
Choline chloride (70%)	—	0.22
Mineral mix no. 1 [6]	2.0	—
Mineral mix no. 2 [7]	—	2.3
$CaHPO_4 \cdot 2H_2O$ [8]	2.2	3.64
$CaCO_3$ [9]	1.0	1.63
Cr_2O_3 bread	1.0	1.0
Fatty acids or glucose, or both [10]	29.0	50.64

[1] ADM C-1 Assay protein, Archer-Daniels-Midland Company, Minneapolis.
[2] Isolated soybean protein continuously extracted with methanol for 48 hours was used in diet B during the fourth week of the experiment when the chicks were fed the pure fatty acids.
[3] Solka-Floc, Brown Company, New York.
[4] Vitamin mix no. 1, Young et al. (11).
[5] Vitamin mix no. 2. Similar to vitamin mix no. 1 except that the niacin was increased to 5 mg and 12.5 mg of ethoxyquin were added/100 g of diet. The choline chloride was removed and the mix was made to 1.0 g/100 g of diet by the addition of glucose.
[6] Mineral mix no. 1. Young et al. (11).
[7] Mineral mix no. 2. Supplies/100 g diet, in g: NaCl, 0.77; KH_2PO_4, 0.2; $KHCO_3$, 0.791; $MgSO_4$, 0.371; $CaCO_3$, 0.098; in mg: $MnSO_4 \cdot H_2O$, 30.0; $FeSO_4 \cdot 7H_2O$, 30.0; $CuSO_4 \cdot 5H_2O$, 3.0; ZnO, 6.0; NaI, 0.209; Na_2SeO_3, 0.022; $NaMoO_4 \cdot 2H_2O$, 0.1.
[8] Diet A contained commercial grade dicalcium phosphate. Diet B contained USP $CaHPO_4 \cdot 2H_2O$.
[9] Diet A contained limestone. Diet B contained AR $CaCO_3$.
[10] Cerelose, Corn Products Company, New York.

supplemented with 2% of safflower oil for 2 weeks. At 14 days the chicks were weighed and those chicks not deviating in weight more than 3 g from the mean were allotted according to weight into equal groups of 4 chicks each. From the fourteenth to twentieth day inclusive, duplicate lots of chicks were fed diet B supplemented with fatty acid mixtures made to specific ratios of palmitic acid to oleic or linoleic acid, using the same fatty acid sources as described for experiments 1 and 2 (table 1, samples A, B, C and D). From the twentieth to the twenty-eighth day the chicks were fed diets containing the same ratio of palmitic acid to oleic or linoleic acid provided by the pure fatty acids (table 1, samples E, F and G).

In all experiments excreta collections were made on each of 4 consecutive days during the fourth week. Each day's collection was frozen, and after final collection the total excreta from each lot were slurried in a Waring Blendor. A proportion of the slurry was frozen and stored at −20°C and the remainder was dried at 65 to 70°C in a forced-air oven. The frozen excreta were used for the determination of the individual fatty acids. Analyses were made on the diets and dried excreta for moisture, total lipid, and chromium. Chromic oxide was incorporated in each diet as an index substance, thus eliminating the need for quantitative collection of excreta. The methods used in collecting samples, and the analysis for total and individual fatty acids, moisture, and chromium, were described by Hill et al. (11), Renner and Hill (12) and Young et al. (10).

RESULTS AND DISCUSSION

Typical analyses of the various sources of the saturated and unsaturated fatty acids are shown in table 1. The ratios of dietary fatty acids resulting from various combinations are shown in tables 3, 4, and 5. The values are expressed as weight ratios of oleic or linoleic acid to the saturated fatty acid, when the latter is given a value of one.

The results from the first 2 experiments were in good agreement, and are therefore combined in tables 3 and 4, since the experimental conditions were similar. The absorbability values of the total fatty acid mixtures, and of the palmitic and stearic acids, are summarized by experiments.

In experiments 1 and 2, the absorbability of oleic acid, whether fed alone or in a mixture of fatty acids, was found to average 94.6%, with a range of 90 to 99%. The average absorbability of linoleic acid was 97.5%, with a range of 94 to 99%. The high absorbability values of these fatty acids were consistent in experiments 1 and 2 regardless of the amount or type of saturated fatty acids present in the mixture. Therefore, these values have not been included in tables 3 and 4.

The absorbability of palmitic acid when fed singly was found to average approximately 25%, regardless of the level fed. This value was significantly higher than

TABLE 3

Absorbability of palmitic acid as influenced by various ratios of oleic and linoleic acid in the diet

Group no.	Exp. no.	Fatty acid in diet [1]	Ratio of unsaturated fatty acids to palmitic when palmitic = 1		Absorbability value			
					Total fatty acids		16:0	
			Oleic	Linoleic	Exp. 1	Exp. 2	Exp. 1	Exp. 2
		%			%	%	%	%
1	1	5.2 P(6.5) [2]	0.026		34		35	
2	2	7.7 P(9.1)	0.025			38		27
3	2	14.7 P(16.6)	0.01			25		25
4	2	21.5 P	0.01			21		23
5	2	15.4 P	0.22			57		53
6	2	17.8 P	0.36			75		66
7	2	8.0 P(14.4)	0.57			76		62
8	2	13.0 P	0.81			87		79
9	1	10.0 P	1.24		87		80	
10	2	10.1 P	1.34			92		85
11	2	6.8 P	2.56			93		89
12	1	5.9 P	2.92		90		84	
13	2	12.1 P	0.1	0.50		59		39
14	2	15.0 P	0.11	0.61		69		52
15	2	15.6 P	0.29	0.29		72		60
16	1	7.1 P	0.35	1.35	89		81	
17	1	6.6 P	0.36	1.79	94		87	

[1] P indicates palmitic acid; S, stearic acid.
[2] Values in parentheses are total amount of fatty acid mixture in diet including lipid material in dietary ingredients. If no value shown, total is 21 to 23%.

TABLE 4

Absorbability of palmitic and stearic acids as influenced by various ratios of oleic and linoleic acid in the diet

Group no.[1]	Exp. no.[1]	Fatty acid in diet [2]	Ratio of unsaturated to saturated fatty acids when saturated = 1	Absorbability value		
				Total fatty acids	16:0	18:0
				Exp. 1	Exp. 1	Exp. 1
		%		%	%	%
18	1	3.8 (6.2) [3]	—	14	—	14
19	1	12.0 P 7.0 S	—	12	12	2
20	1	3.0 P 5.2 S	(O) [2] 1.4	67	52	18
21	1	3.3 P 3.8 S	(O) 2.0	78	46	49
22	1	4.0 P 4.0 S	(O+L) 1.9 (1:4.5) [4]	78	77	17
23	1	3.5 P 3.3 S	(O+L) 2.5 (1:4.9) [4]	87	79	41
24	2	9.3 P 2.0 S	(O+L) 0.9 (1:0.23) [4]	79	68	44

[1] Continued from table 3, and refers to same experiments as table 3.
[2] Fatty acids denoted: P = palmitic; S = stearic; O = oleic; L = linoleic acid.
[3] See footnote 2, table 3.
[4] Figure in parentheses represents ratio of oleic to linoleic acid in the unsaturated fatty acid portion.

TABLE 5

Absorbability value of pure fatty acids when fed to chicks consuming a purified diet

Group no.	Fatty acid in diet [1]	Ratio of unsaturated fatty acids to palmitic when palmitic = 1		Absorbability value			
		Oleic	Linoleic	Total fatty acids	16:0	18:1	18:2
	%			%	%	%	%
1	14.5 P	—	—	5.5	2.4	—	—
2	11.3 O	—	—	78.1	—	74.7	—
3	11.2 L	—	—	66.8	—	—	66.2
4	17.2 P	0.26	—	28.1	15.0	75.5	—
5	15.1 P	0.43	—	40.6	25.6	74.5	—
6	12.8 P	0.69	—	57.5	35.8	85.9	—
7	17.7 P	—	0.26	24.4	9.1	—	82.9
8	15.4 P	—	0.41	39.9	20.2	—	86.1
9	11.1 P	—	0.95	49.2	20.5	—	78.2

[1] P indicates palmitic acid; O, oleic acid; L, linoleic acid.

the zero to 10% absorbability observed by Renner and Hill (1) and Renner.[3] The higher value observed in these experiments may be due in part to the use of a laboratory that was fumigated just prior to the experiment, and to the use of a higher protein diet than that used by Renner and Hill (1). Young et al. (10) observed that both these factors influenced absorbability of fatty acid mixtures high in saturated fatty acids.

Increasing the ratio of oleic acid to palmitic acid (groups 5 through 12, table 3) resulted in a stepwise increase in absorbability of the total fatty acid mixture. Gas chromatographic analysis of the dietary and fecal lipids showed that the increased absorbability of the mixture was due not only to the presence of the well utilized oleic acid, but also to a significant improvement in the absorbability of the palmitic acid. Small additions of oleic acid caused a marked improvement in the absorbability of palmitic acid. When sufficient oleic acid was present in the mixture, the absorbability of the palmitic acid was increased to 80% or more.

Chicks in groups 13 through 17 (table 3) were fed fatty acid mixtures to determine the effect of linoleic acid on the absorbability of palmitic acid. The hydrolyzed safflower oil used as a source of linoleic acid also contained a small amount of oleic acid (table 1, sample D). Consequently, in groups 13, 14, 16 and 17 the final fatty acid mixtures contained a small amount of oleic acid in addition to the linoleic acid (table 3). Group 15 was fed a mixture in which the oleic and linoleic acid were formulated to be equal. In addition the sum of the oleic and linoleic acids in this group equaled the total amount of unsaturated fatty acid which was predominantly linoleic acid in group 13.

There was a significant improvement in the absorbability of palmitic acid from those mixtures that contained 2 unsaturated fatty acids. However, based on the effect of oleic acid itself on absorbability of palmitic acid (groups 5 through 12), the magnitude of the improvement in groups 13, 14 and 15 appears to be directly proportional to what would be expected from the amount of oleic acid provided in these mixtures. Therefore in these 3 groups the presence of linoleic acid in addition to the oleic acid did not appear to provide a further improvement in the absorbability of the palmitic acid.

Chicks in groups 16 and 17 were fed diets containing significantly higher ratios of linoleic to palmitic acid. Again the hydrolyzed safflower oil provided sufficient oleic acid to increase the absorption of the palmitic acid to 66% (compare with group 6). Therefore it appears that the large amount of linoleic acid increased the absorbability of palmitic acid from 66% up to 81 or 87%.

The absorption of stearic acid when fed singly and in combination with other fatty

[3] See footnote 2.

acids is shown in table 4. These results were obtained in the same experiments as those shown in table 3, and therefore the 2 tables are directly comparable. Stearic acid by itself was absorbed only to 14%. When a mixture of stearic acid and palmitic acid was fed, the absorption of both fatty acids was reduced to 2 and 12%, respectively.

The addition of oleic acid to a mixture of palmitic and stearic acid (groups 20 and 21) improved the absorbability of both saturated fatty acids. However, the degree of improvement in the absorbability of palmitic acid when fed in the presence of stearic acid was not as great as that obtained when palmitic acid was fed as the only saturated fatty acid (groups 10, 11 and 12, table 3). Increasing the amount of oleic acid in the mixture of palmitic and stearic acid improved the absorption of stearic acid but did not improve the absorption of palmitic acid beyond the initial increase.

In groups 22, 23 and 24 the unsaturated fatty acid fraction contained both oleic and linoleic acid in the ratios shown in table 4. Under conditions when the unsaturated fatty acid mixture was primarily linoleic acid, the absorption of the palmitic acid was significantly better than from those mixtures containing oleic acid as the only unsaturated fatty acid (groups 20 and 21). However, the absorption of stearic acid in groups 22 and 23 was not improved by the linoleic acid. Group 24 shows the absorption of palmitic and stearic acid when palmitic acid makes up the greatest proportion of the saturated fatty acids and the unsaturated fatty acid portion is predominantly oleic acid. The absorbability of both saturated fatty acids is higher than that observed in group 20 and almost equal that obtained in group 23.

The results show that the absorbability of palmitic and stearic acid from any given mixture of fatty acids is influenced not only by the ratio of these saturated fatty acids to oleic or linoleic acid, or both, but also to the ratio of palmitic to stearic acid within the mixture.

In figure 1 the average 4-week chick weight and feed efficiency data for each treatment in experiments 1 and 2 have been plotted against the absorbability value of the total fatty acid mixture. Those groups of chicks consuming diets containing large amounts of the saturated fatty acids showed a marked depression in growth rate, and poor feed efficiency. The chicks consuming diets containing the fatty acid mixtures that had high absorbability values showed good growth. A linear relationship was observed between percentage absorbability of the fatty acid mixture and the efficiency of feed conversion. However, both growth and feed efficiency were found to be highly variable when the absorbability of the fatty acid mixture was less than 30 to 40%.

In experiment 3, fatty acids of high purity (table 1) were combined in specific ratios and fed to chicks in a purified diet. Analyses of experimental diet B (table 2) showed that it contained 0.07% total lipid. A preliminary experiment was conducted to determine whether chicks fed this purified diet would show satisfactory absorption of fatty acids. It was found that lard fatty acids had an absorbability value of 73%. This was lower than the absorbability values of 81 to 88% observed for lard fatty acids fed in a diet similar to diet A, table 2 (10). It was therefore expected that the absorbability values for either the pure fatty acids or the various ratios of palmitic to oleic and palmitic to linoleic acid would be low. However, the values found were lower than anticipated and lower than the values observed in experiments 1 and 2. It is not known whether the low values found in this experiment were due to the feeding of the pure fatty acids themselves or to the lack of some component in either the hydrolyzed oils or natural feed ingredients which is necessary for increased absorption of these fatty acids.

In experiment 3 (table 5) the palmitic acid when fed alone was virtually unutilized by the chicks.

The absorbability of oleic acid was significantly lower than the value of 94% observed by Renner and Hill (1). The absorption of both the pure oleic acid and the linoleic acid fed in this experiment was lower than the absorption of these 2 fatty acids when supplied as hydrolyzed olive oil and safflower oil (experiments 1 and 2,

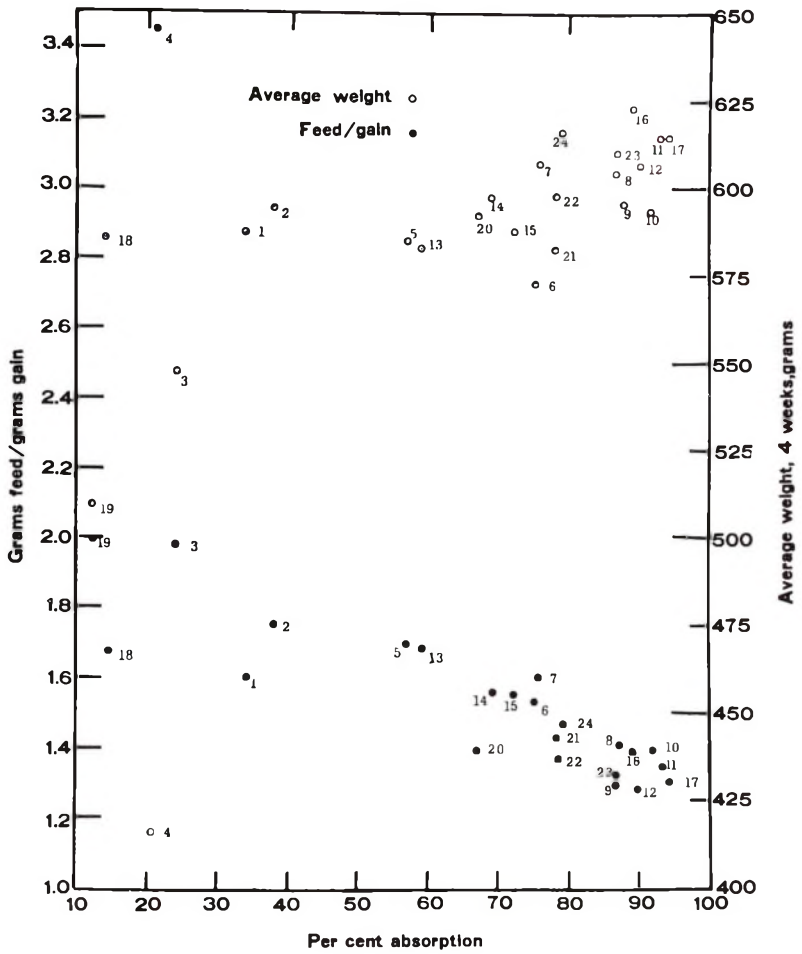

Fig. 1 Chick growth and feed conversion related to percentage absorption of the fatty acid mixture (subscript numbers refer to group numbers in tables 3 and 4).

Although the absolute absorbability value for palmitic acid was low the relative improvement with each additional increment of oleic acid was of about the same magnitude as that observed in the previous experiments. The addition of linoleic acid to palmitic acid also increased the absorbability of the palmitic acid. However, this improvement was not as great as that obtained from oleic acid and was not improved beyond a maximum of 20% when with the highest level of linoleic acid. The absorbability of the linoleic acid itself was significantly better when fed in a mixture of palmitic acid than when fed alone.

DISCUSSION

Recent reports in the literature indicate that at least 2 phenomena may be involved in the mechanism of fatty acid absorption. The first is a transformation in the lumen of the fatty acid to a physical-chemical form which is more readily solubilized and made available for absorption.[4] The second is the esterification of the fatty acid within the intestinal mucosa to form triglycerides which appear in the lymphatic chyle (13–20).

[4] Hofmann, A. F., and B. Borgstrom 1962 Physicochemical state of lipids in intestinal contents during their digestion and absorption. Federation Proc., 21(1): 43 (symposia).

Hofmann and Borgstrom[5] have proposed that prior to absorption from the lumen the monoglycerides and fatty acids from the hydrolysis of fats form mixed micelles with the bile acids. In vitro experiments with the micelles of bile acids and monoolein increased the solubilization of palmitic and stearic acid. Oleic acid also forms micelles with bile salts which will solubilize the saturated fatty acids. However, micelles made up of bile salts and oleic acid are not as effective as micelles containing monoolein.[6]

Numerous studies have been made on the mucosal enzyme systems responsible for the esterification of fatty acids to glycerides. Homogenates of rat intestinal mucosa or mitochondrial preparation from the intestinal mucosa of the rabbit have shown that the resynthesis of triglycerides in the intestinal mucosa is dependent on ATP, coenzyme A, reduced glutathione and magnesium ions (21, 22). Clark and Hubscher (22) observed that a mitochondria preparation required either α-glycerophosphate or monoglyceride as an acceptor.

Senior and Isselbacher (23) have shown the presence of an enzyme monoglyceride acylase which catalyzed the condensation of palmityl coenzyme A and monoglyceride to form a diglyceride in rat mucosa cells. The highest activity was noted in the microsomal fraction of the mucosal cells. In their experiments the mitochondria fraction was less active. The reaction did not require ATP; therefore, the authors concluded that the ATP requirement observed by other investigators (21, 22) was probably needed in the formation of the fatty acyl CoA.

It is assumed that fatty acid absorption in the chick is similar to that of other species. However, the presence of the enzyme system in the endoplasmic reticulum or mitochondria of the mucosal cells as shown by in vitro studies does not assure the maximal absorption of fatty acids under all conditions. This is particularly true in the chick, where stearic and palmitic acids are very poorly utilized when fed singly. In addition to the effect of micelle formation it is possible that the improved absorption of the 2 saturated fatty acids in the presence of oleic acid may be a preferential synthesis of monoolein in the brush border of the mucosal cells and this monoglyceride then acts as the acceptor of the saturated fatty acids in the formation of di- and triglycerides. This hypothesis is supported by the observation of Clark and Hubscher (25) that monoolein in their in vitro system incorporated more (1-C^{14}) palmitate than did monolaurate or monopalmitin. However, Senior and Isselbacher (23) observed no difference between either the α- or β- isomers of monopalmitin or monoolein in their ability to act as acceptors for palmityl-CoA to form diglycerides in their in vitro system. A recent study with rats[7] showed that glycerol-mono-linoleate or monooleate increased the absorption of cholesterol-4-C^{14} to a greater degree than did monostearate.

Renner[8] has shown that chicks fed palmitic acid in a mixture with triolein showed a stepwise improvement in absorption of the palmitic acid up to 49% when the palmitic acid to triolein ratio was 1:3. In this case it appears that the pancreatic lipase hydrolyzes the triolein to oleic acid and monoolein and that both the formation of a micelle and the synthesis or absorption, or both, of monoolein as an acceptor are involved in the improved absorption of palmitic acid.

The reason that linoleic acid is not as effective as oleic acid in improving the absorbability of palmitic acid is not known. It appears that the improvement in absorbability of the saturated fatty acid cannot be attributed simply to a decrease in melting point due to the presence of an unsaturated fatty acid. Therefore oleic acid must play a more direct role in the absorption process, either in the lumen or in the mucosa cells, to facilitate the increased absorption of palmitic acid.

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[5] See footnote 4.

[6] Hofmann, A. F., personal communication.

[7] Pinter, K. G., O. N. Miller and J. G. Hamilton 1963 Effect of monoglycerides on absorption of cholesterol and turnover rate of cholesterol esters. Fed eration Proc., 22: 376 (abstract).

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Periodic B-vitamin Supplementation of Growing Rats Fed a Vitamin-free Diet

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ABSTRACT The effects on rats fed a vitamin-free diet orally supplemented with a complete array of B-vitamins at various intervals of time were investigated. Feed intakes, weight gains and final body fat percentages declined as the interval between successive vitamin doses was increased. Reduced partial efficiencies of feed utilization with decreasing frequency of vitamin administration indicate a metabolic restriction in vitamin availability as the interval between doses is increased. With diets lacking thiamine, pyridoxine or both vitamins, the provision of the missing vitamin or vitamins at intervals of 4 days was not adequate to maintain feed intakes and weight gains of rats. Paired-feeding trials indicate that, apart from a metabolic restriction in vitamin availability, the periodic provision of B-vitamins to rats has a considerable depressant effect on feed intakes.

It has long been known that rats fed a flavin-deficient diet supplemented at weekly intervals with single doses of riboflavin can grow to adult size, although growth proceeds at a slightly subnormal rate (1). For a number of vitamins is has been shown that oral supplementation apart from the diet results in reduced growth rates of rats as the interval between successive doses with the vitamin was increased (2–4). Since vitamin interactions are known to occur (5–7), the study of individual vitamins under conditions of periodic administration may be complicated by this factor. It is clear, that elimination from the diet of a single vitamin results in an imbalance between vitamins, whereas the periodic administration of the missing vitamin also causes a temporary change in the relationships between the vitamins.

It was, therefore, pertinent to determine whether a growth depression would still occur when a complete array of B-vitamins was administered to rats apart from the diet and at various intervals of time.

EXPERIMENTAL

Male, weanling Sprague-Dawley rats, obtained commercially,[2] were used in all trials. The rats were housed in cages with wire bottoms and kept in a room maintained at 26 ± 1°C. Each rat had water available at all times.

The basic, vitamin-free ration was composed of the following parts by weight: vitamin-free casein,[3] 25; mineral mixture,[4] 4; sucrose, 65; and cottonseed oil, 5. To obtain a vitaminized ration, B-vitamins[5] were added to this basic diet at the level used by Meyer and Hargus (8). All diets were stored in a refrigerator.

The same B-vitamins, in the same proportions and including choline, were made up in distilled water to such a concentration that a known number of drops provided the same amounts of vitamins as present in 10 g of the vitaminized diet. This solution was used for the oral dosage of the rats, by means of a pipet. The vitamin solution was made up fresh in small amounts at frequent intervals and kept in a refrigerator. Ample amounts of vitamins A, D, E and K were administered to all rats at weekly intervals.

At the end of each experiment carcass composition of each rat was determined according to the method described by Meyer (9).

Experiment 1. A total of 60 rats was distributed into 2 comparable groups of 30 animals each. One of these groups was

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[4] Phillips and Hart, J. Biol. Chem., *109*: 657, 1935.

[5] One per cent of the vitamin mix was added to the ration. The vitamin mix contained the following vitamins in milligrams per 100 g: thiamine, 30; riboflavin, 30; pyridoxine, 20; niacin, 200; Ca patothenate, 200; folic acid, 3; biotin, 1; and vitamin B_{12}, 0.2. Sucrose was added to equal 100 g. Choline chloride was added at 0.1% of the ration.

fed ad libitum, the other for 2 daily periods of 4 hours each. Within each group, 6 treatments of 5 rats each were formed and designated treatments A to F, respectively. With treatment A the complete diet was fed and for all other treatments, the vitamin-free diet. With treatment B each rat was given its dose of B-vitamins in 2 equal daily portions and with treatment G, rats received the same amount of B-vitamins in single daily administrations. For treatments D to F, respectively, the vitamins were given at 2-, 4- and 8-day intervals. For treatments B to F, inclusively, the total dosage with B-vitamins was the same.

Weekly feed intakes and weight gains were recorded for each rat, and after 32 days on trial, the experiment was terminated.

Experiment 2. This was a repetition of experiment 1 with 4 rats/treatment at each feeding level. The vitaminized ration was made up in small amounts at frequent intervals to eliminate possible losses of vitamins during storage.

Experiment 3. Six comparable groups of 5 rats each were allocated to the treatments given in table 5. Weekly weights were recorded for each rat, and for treatments 1 to 4, weekly feed intakes were determined. For treatments 5 and 6, feed intakes were measured twice daily.

To eliminate the factor of variable feed intakes, 2 groups of 5 rats each were pair-fed the complete diet in the same amounts as consumed by the respective pair-mates on treatments 5 and 6. Pairing was on an initial live weight basis, and after 3 weeks on trial the experiment was terminated.

RESULTS AND DISCUSSION

The results of the first 2 experiments are presented in tables 2 and 3. Data for treatment A of experiment 1 were excluded since vitamin losses during storage of this diet were suspected.

In both trials, the ad libitum feeding regimen resulted in significantly greater feed intakes ($P < 0.01$) than obtained when feeding time was limited to 8 hours/day. Feed intakes in both trials were significantly influenced ($P < 0.01$) by the frequency with which the vitamins were given, intakes declining with decreasing frequency of vitamin administration. In experiment 2, feed intakes were also dependent upon an interaction effect between the frequency of vitamin dosage and the feeding regimen ($P < 0.01$).

The weight gains by the rats in both trials were significantly influenced by the frequency of oral B-vitamin supplementation ($P < 0.01$) and declined as the interval between vitamin doses was increased. For experiment 2, an interaction effect between vitamin dosage and feeding regimen also affected the weight gains ($P < 0.05$).

The frequency of vitamin dosage influenced the final body fat percentages of the rats at the 1 and 5% levels of significance in trials 1 and 2, respectively. In experiment 1, ad libitum feeding yielded greater body fat percentages ($P < 0.01$) than observed when feeding for only 8 hours daily.

Both weight gains and final body fat percentages were related to feed intakes. Since feed intakes decreased with decreasing frequency of vitamin administration, reduced weight gains and body fat percentages may be expected under these conditions. The role of B-vitamins in influencing feed intakes thus appears to be of considerable importance.

The reduction in feed:gain ratios with increasing intervals between successive doses of B-vitamins suggests a limitation

TABLE 1

Feed intakes, weight gains and final body fat percentages of rats fed a vitamin-free diet ad libitum or a vitaminized diet in equivalent amount

	Diet	
	Vitamin-free	Vitaminized
Avg feed intake, g[1]	78.1 ± 2.6	78.1 ± 2.6
Avg weight gain, g[1]	−6.6 ± 2.3	−7.6 ± 1.5
Avg final body fat, %[1]	4.6 ± 0.5	4.2 ± 0.3

[1] Mean ± SE of mean.

TABLE 2

Effect of frequency of oral B-vitamin administration on growing rats (exp. 1)

Treatment	B-vitamin provision	Avg feed intake[1]	Avg weight gain[1]	Avg final body fat[1]	Feed:gain ratio
		g	*g*	%	
		Ad libitum feeding			
A	In the diet	—	—	—	—
B	Twice daily	388 ± 5.7[a]	160 ± 1.9[a]	13.2 ± 0.5[a]	2.42
C	Once daily	338 ± 11.4[b]	140 ± 3.8[a,b]	9.3 ± 1.2[b]	2.41
D	Every 2 days	326 ± 12.6[b]	120 ± 5.9[b]	10.4 ± 0.7[b]	2.73
E	Every 4 days	331 ± 7.8[b]	126 ± 4.2[b]	10.7 ± 0.6[b]	2.63
F	Every 8 days	255 ± 17.6[c]	84 ± 7.4[c]	8.9 ± 1.3[b]	3.02
		Twice daily feeding			
A	In the diet	—	—	—	—
B	Twice daily	303 ± 10.4[a]	139 ± 3.2[a]	9.7 ± 0.7[a]	2.17
C	Once daily	260 ± 2.1[b,c]	117 ± 2.0[a,b]	8.2 ± 0.1[a]	2.22
D	Every 2 days	293 ± 13.6[a,b]	126 ± 10.0[a,b]	8.8 ± 0.7[a]	2.32
E	Every 4 days	246 ± 5.1[c,d]	109 ± 3.6[b,c]	9.2 ± 0.6[a]	2.26
F	Every 8 days	212 ± 7.4[d]	70 ± 11.1[d]	6.1 ± 0.7[b]	3.04

[1] Mean ± SE.
[a—d] Within feeding regimens, means in the same column and having the same letter superscript do not differ statistically from each other ($P < 0.05$).

TABLE 3

Effect of frequency of oral B-vitamin supplementation on growing rats (exp. 2)

Treatment	B-vitamin provision	Avg feed intake[1]	Avg weight gain[1]	Avg final body fat[1]	Feed:gain ratio
		g	*g*	%	
		Ad libitum feeding			
A	In the diet	428 ± 35.4[a]	156 ± 14.2[a]	12.1 ± 2.2[a]	2.71
B	Twice daily	386 ± 23.0[a]	145 ± 6.1[a]	10.3 ± 1.9[a,b]	2.66
C	Once daily	328 ± 14.8[b]	108 ± 5.5[b]	9.0 ± 1.1[a,b]	3.05
D	Every 2 days	283 ± 16.2[b,c]	94 ± 9.8[b]	10.0 ± 1.6[a,b]	3.02
E	Every 4 days	239 ± 15.9[c,d]	66 ± 12.8[c]	6.5 ± 1.3[b]	3.62
F	Every 8 days	198 ± 6.9[d]	37 ± 4.7[d]	9.2 ± 0.8[a,b]	5.35
		Twice daily feeding			
A	In the diet	265 ± 25.5[a,b]	108 ± 9.3[b,c]	9.1 ± 1.9[a,b]	2.44
B	Twice daily	303 ± 18.0[a]	128 ± 7.2[a,b]	10.0 ± 1.5[a]	2.38
C	Once daily	314 ± 8.8[a]	132 ± 2.9[a]	10.0 ± 1.4[a]	2.38
D	Every 2 days	244 ± 12.2[b]	91 ± 2.3[c]	9.8 ± 0.8[a,b]	2.67
E	Every 4 days	181 ± 8.5[c]	59 ± 4.3[d]	5.9 ± 1.2[b]	3.05
F	Every 8 days	170 ± 3.7[c]	35 ± 6.5[e]	6.6 ± 1.0[a,b]	4.80

[1] Mean ± SE.
[a—e] Within feeding regimens, means in the same column and having the same superscripts are not statistically different from each other ($P < 0.05$).

on the availability of vitamins for metabolic processes when provided only at intervals. However, such ratios have to be interpreted with caution since they necessarily increase as maintenance feed intakes are approached.

Since 2 feeding regimens were involved in these 2 trials, it is possible to calculate the partial efficiency of feed utilization for weight gain for the various treatments. This gives an efficiency of feed utilization independent of feed intake relative to maintenance requirements. Partial efficiencies are obtained as the ratios of the differences in weight gains to differences in feed intakes for the same treatment on the 2 feeding

regimens. According to Kleiber (10), a diet is deficient in a nutrient whose addition increases the partial efficiency of the ration. Thus, reductions in the partial efficiency of a diet can be used as an indication of a nutrient deficiency effect on metabolic processes. Although these values could not be calculated in every case and varied for the same treatment in the 2 experiments, a tendency existed for partial efficiencies to be reduced as the vitamin doses were spaced further and further apart. For treatment B, partial efficiencies were 24.5 and 21.0% in experiments 1 and 2, respectively, but only 19.8 and 11.6%, respectively, for treatment E. A reduction in the availability of vitamins for metabolic processes is, thus, indicated but the mechanism involved whereby such a reduction is achieved remains obscure. Poor absorption of large doses of vitamins or inefficient storage in the body after absorption may be involved (11). The effects of feed intakes on weight gains and body composition of rats under different conditions are presented in tables 1 and 4. The data of table 1 show that with identical feed intakes of a vitamin-free or a complete diet, similar weight losses were experienced by rats and that body fat percentages were also similar. The rats fed the complete diet were physically more active than pair-mates fed the vitamin-free diet and this probably accentuated their weight loss since increased activity would serve to increase the maintenance require-

TABLE 4

Effects on rats of administering B-vitamins every 4 days or including them in the diet under conditions of equalized feed intakes

	Provision of B-vitamins	
	Oral, once/4 days	Included in diet
Avg feed intake, g[1]	134.0 ± 5.6	134.0 ± 5.6
Avg weight gain, g[1]	29.7 ± 2.4	40.8 ± 3.8
Avg final body fat, %	5.3 ± 0.4	7.6 ± 0.7
Feed:gain ratio	4.51	3.28

[1] Mean ± SE of mean.

TABLE 5

Effect of B-vitamin supplementation and of thiamine, pyridoxine or both vitamins on the ad libitum feed intakes and weight gains of growing rats

Treatment	B-vitamin administration	Avg feed intake[1]	Avg weight gain[1]	Avg final body fat[1]	Feed:gain ratio
		g	g	%	
1	Included in the diet	276 ± 9.6[a]	114 ± 2.7[a]	10.5 ± 1.1[a]	2.41
2	Vitamin B_1-free diet; vitamin B_1 given orally every 4 days	234 ± 4.3[b]	86 ± 4.6[b]	8.7 ± 1.0[a,b]	2.71
3	Vitamin B_6-free diet; vitamin B_6 given orally every 4 days	212 ± 6.0[b,c]	75 ± 5.7[b,c]	8.2 ± 0.5[a,b]	2.82
4	Vitamin B_1- and B_6-free diet; both vitamins given orally every 4 days	196 ± 7.6[c]	70 ± 5.6[c]	6.6 ± 0.4[b,c]	2.80
5	Vitamin-free diet; all B-vitamins given orally every 4 days	134 ± 5.6[d]	30 ± 2.4[d]	5.3 ± 0.4[c]	4.51
6	Vitamin-free diet; no B-vitamins given	78 ± 2.6[e]	−7 ± 2.7[e]	4.6 ± 0.5[c]	—

[1] Mean ± SE.

[a—e] Within columns, different superscripts differ from each other at the 1% level of significance.

ments of these rats. The data suggest reduced feed intakes as the main cause of reduced weight gains.

On the other hand, when feeding a complete diet in the same amount as a vitamin-free diet supplemented with B-vitamins every 4 days, rats on the former treatment gained significantly more weight ($P < 0.01$) and accumulated more body fat ($P < 0.05$) than pair-mates fed the latter diet (table 4). In this trial an increase in feed intakes invariably followed an oral vitamin dose, confirming the influence of vitamins on feed intakes. However, the fact that with equal feed intakes rats fed the complete diet gained more weight and body fat than pair-mates fed a vitamin-free diet orally supplemented with B-vitamins every 4 days, is evidence that a metabolic deficiency of vitamins was experienced by the rats on the latter treatment. It may, thus be concluded that at least 2 factors, viz., the effect of the B-vitamins both on feed intakes and on satisfying metabolic needs, play a role in determining weight gains and fattening in rats.

The individual and combined effects of thiamine and pyridoxine under conditions of periodic, oral administration were examined in experiment 3 (table 5). The results show that rats fed diets deficient in thiamine, pyridoxine or both and given the lacking vitamin or vitamins at intervals of 4 days exhibit reductions in feed intakes and weight gains as well as body fat percentages. The decreases in feed intakes and weight gains, however, were significantly less than observed with rats fed a vitamin-free diet orally supplemented with all B-vitamins at the same intervals of time. Therefore, apart from thiamine and pyridoxine, singly or in combination, other vitamins must be involved in affecting feed intakes and weight gains of rats.

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Vitamin E Content of Vegetable Oils and Fats[1]

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ABSTRACT Analyses of vegetable oils or fats from 17 different plant types showed as low as zero μg of α-tocopherol/g of castor bean and linseed oils and as high as 1276 μg/g of wheat germ oil. Contents of total tocopherol ranged from 2 μg/g of coconut oil to 1896 μg/g of wheat germ oil. In individual types, the tocopherol levels appeared to be influenced by source of plant, time of harvest, stability after harvest, refining procedure, and perhaps by commercial hydrogenation procedures. Estimates of man's requirement for vitamin E as related to the dietary level of polyunsaturated fatty acids suggest that among the common edible polyunsaturated oils (cottonseed, corn, safflower, and soybean), only cottonseed supplies sufficient vitamin E to counterbalance the effect of its polyunsaturated fatty acids.

The last few years have witnessed a widespread and remarkable popularization of the idea that hypercholesterolemia can and should be treated by increasing the dietary intake of polyunsaturated fatty acids. The Council on Foods and Nutrition of the American Medical Association has generally supported this concept in a recent guide for physicians (1).

A general relationship between polyunsaturated fatty acids and vitamin E was observed years ago. Recent reports are more specific, however, and direct attention to the extent of increase in dietary requirement for vitamin E with an increase in dietary polyunsaturated fatty acids in man (2), the monkey (3), the rat (4), and the chick (5, 6).

No reports have appeared recently to provide guides for the content of tocopherols, and specifically of α-tocopherol — the most active analogue — in vegetable oils consumed in appreciable quantities by the American public. The most recent and comprehensive survey of the vitamin E content of foods (7) was carried out prior to the advent of the more accurate and definitive column chromatographic (8, 9) and paper chromatographic (10, 11) analytical methods now available. The survey of feedstuffs by Ames (12) included some results obtained by these latter methods, but the number of oils was limited and the data were obtained on oils available in England.

We report here the content of total and α-tocopherol observed in vegetable oils analyzed on occasion during the last 4 years. We have concentrated on the most common oils but have included values obtained on less common and even inedible plant oils. We have also included some results which suggest changes in tocopherol content and distribution during various processing steps.

METHODS AND MATERIALS

Sources of materials. Fats and oils were obtained from local grocery stores, from basic producers, and from wholesale distributors. Corn samples were obtained from various parts of the United States and also from England.[2]

Preparation of samples. In most cases the samples were used as obtained. Fat was recovered from margarine by extraction with petroleum ether.[3] Lipids were extracted from corn with ethanol or ether as described by Quaife and Harris (13). Ethanol usually extracted slightly more lipid, but not more tocopherol, than ether.

Saponification. Saponification of a weighed sample of fat or oil (minimum of 1 g) and recovery of the nonsaponifiable material were carried out as described by

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[1] Communication no. 305, Research Laboratories, Distillation Products Industries.

[2] Courtesy of Dr. I. M. Sharman, Cambridge, England.

[3] Skellysolve F, Skelly Oil Company, bp 35 to 60°C.

TABLE 1

Tocopherol content of oils and fats from various plants

Oil	State	Supplier	Samples	Total tocopherols	α-Tocopherol		Others present
				μg/g	μg/g	%	
Castor bean	refined	A	1	291	0	0	?, γ or β, δ
Cocoa butter	refined	B	3	155 (136–175)	26 (16–31)	17 (9–22)	γ or β
Coconut	refined	C	3	11 (2–16)	5 (2–6)	45	γ
	refined	D	1	24	16	67	γ, η
Corn[1]	refined[2]	E	4	427 (295–636)	194 (147–236)	47 (37–51)	γ, δ in 1
	refined[2]	F	1	661	157	24	γ
	refined, partially hydrogenated[2]	G	1	378	177	47	γ
	refined, partially hydrogenated[2]	H	1	571	169	30	γ
Cottonseed	refined[2]	I	3	611 (564–618)	375 (338–434)	61 (55–67)	γ
	refined[2]	J	2	298 (259–336)	182 (172–192)	62 (57–66)	γ
	refined[2]	H	1	568	352	62	γ
	refined[2]	K	1	635	360	57	γ
	crude	L	1	344	230	67	γ
	crude, hydrogenated	M	1	201	102	51	γ
Grapeseed	refined	N	1	194	149	77	no other spot
Linseed	unknown	O	1	61	0	0	?, γ or β
	unknown	P	1	428	0	0	?, γ or β
Mustard seed	refined	Q	1	446	138	31	γ, trace δ
Olive	refined	R	2	46 (46–46)	31 (30–32)	67 (65–70)	γ
	unknown[2]	H	1	146	124	85	γ
	unknown[2]	S	1	110	78	71	γ
Peanut	refined[2]	T	3	215 (211–217)	130 (128–134)	61 (59–62)	γ
Rapeseed	refined	U	1	433	170	39	γ
Rice bran	crude	U	1	444	264	59	γ or β, η
Safflower seed	refined	V	6	340 (323–363)	308 (284–334)	91 (87–92)	γ

TABLE 1 (*Continued*)

Tocopherol content of oils and fats from various plants

Oil	State	Supplier	Samples	Total tocopherols	α-Tocopherol		Others present
				μg/g	μg/g	%	
	refined[2]	D	2	334 (288–381)	307 (254–360)	91 (88–94)	γ
	refined[2]	W	1	248	226	91	γ
	refined	X	1	492	426	87	γ
	refined, partially hydrogenated[2]	Y	2	232 (169–296)	188 (136–241)	80 (80–81)	γ
	crude	W	1	453	411	91	γ
	crude, solvent extracted	D	1	411	371	90	γ, trace δ
	crude, pre-pressed	D	1	311	290	93	γ
Soybean	refined[2]	Z	3	797 (605–918)	175 (110–242)	21 (18–26)	γ, δ
	refined	AA	1	871	136	16	γ, δ
Sunflower seed	crude	D	1	271	224	83	γ
Tung	best grade	U	1	810	243	30	γ or β
Wheat germ	crude	BB	1	1897	1276	67	β
	unknown	CC	1	1656	848	51	β

[1] See also values in table 3.
[2] Table grade materials obtained from grocery store.

the Analytical Methods Committee (10), except that the ether extract of nonsaponifiable material was dried before evaporation by adding anhydrous Na_2SO_4, instead of after evaporation by adding ethanol and benzene and re-evaporating. The residues were dissolved in petroleum ether.[4]

Although we have not observed destruction of tocopherols by the level of iron (0.0005%) in the Na_2SO_4 which we use, the warning of the Analytical Methods Committee (10) should be heeded and each lot of Na_2SO_4 should be tested for such an effect.

Chromatography. The preferred methods for analysis of tocopherols are based on column chromatography on $MgHPO_4$ (8, 9), paper chromatography (10, 11), and gas-liquid chromatography (14).[5,6] This last technique is relatively recent and has not been used extensively, but it appears to require rigid exclusion of sterols.

We have examined both column and paper chromatography. In general, chromatography on paper is more sensitive and provides better separation of the tocopherols and their unsaturated analogues, termed "tocotrienols" by Bunyan et al. (15). Chromatography on $MgHPO_4$ is less subject to interference from other constituents of the nonsaponifiable fraction, however, and is more rapid for routine determinations of α-tocopherol.

[4] See footnote 3.
[5] K. K. Carroll 1962 Gas-liquid chromatography of fat-soluble vitamins. 36th Fall Meeting, American Oil Chemists' Society, p. 43 (abstract).
[6] P. P. Nair, and D. A. Turner 1962 The application of gas chromatography to the determination of vitamins E and K. 36th Fall Meeting, American Oil Chemists' Society, p. 34 (abstract).

Since our primary objective has been to determine the biologically active tocopherols, we have used the latter procedure for the results reported herein. The only tocopherols, other than α-tocopherol, with significant biological activity are β-, ζ_1-, and ζ_2-tocopherols (see (15)); and these occur to only a limited extent.

The $MgHPO_4$ adsorbent must be checked for suitability as described by Bro-Rasmussen and Hjarde (8). The tocopherols are eluted by successive fractions of 250 ml each of 2, 4, and 6% diethyl ether in petroleum ether.[7] These fractions then contain, respectively, the α- (plus ζ_1- and ζ_2- if present), the β- and γ- (plus ε-, if present) and the δ- (plus η-, if present). Both solvents should be purified by redistillation from KOH pellets, adding aluminum for the diethyl ether and zinc for the petroleum ether.

Resolution by $MgHPO_4$. As part of our evaluation of these analytical procedures, we have routinely examined the eluates from the columns by paper chromatography (10). Except for the occasional materials containing the ζ-tocopherols, this procedure is not necessary for routine determinations of α-tocopherol. It is necessary, however, for determination of total tocopherols if any of the solvent fractions contains more than one tocopherol. The various tocopherols show different degrees of reaction with colorimetric reagents, and an overall factor cannot be applied. In such cases, the tocopherols must be eluted from the paper and analyzed individually to determine the relative amount of each.

Colorimetric measurement. The eluates from the column are evaporated under nitrogen to a volume of 25 ml. Appropriate aliquots are diluted with petroleum ether [8] to give a total volume of 4 ml, and a 4-ml portion of ethanol is then added. Further additions of 1 ml of 0.5% 2,2′-bipyridine in ethanol (w/v) and 1 ml of 0.2% $FeCl_3$ in ethanol (w/v) are followed by measurement of color intensity in an Evelyn colorimeter after 2 minutes for all tocopherols except δ-tocopherol, which is measured after 10 minutes.

Amounts of tocopherol are calculated with factors determined with pure tocopherols.

RESULTS AND DISCUSSION

Table 1 lists the results obtained on 60 samples of fats and oils from 17 different plants. Total tocopherols range from 2 to 24 μg/g in coconut oil to 1656 to 1897 μg/g in wheat germ oil. Levels of α-tocopherol vary, from little or none in coconut oil, cocoa butter, castor bean oil, and linseed oil, up to 848 to 1276 μg/g in wheat germ oil. The percentage of α-tocopherol in total tocopherols varies from 0% in castor bean and linseed oils to about 90% in safflower seed oil.

The percentage of α-tocopherol in total tocopherols is relatively constant for each plant except corn. The content of α-tocopherol and of total tocopherols in samples from a single supplier shows remarkable consistency in some cases (e.g., peanut oil), but appreciable variation in others (e.g., corn oil from supplier E). Samples of a given oil (e.g., cottonseed) from several suppliers usually show appreciable variation in tocopherol contents, perhaps due at least partially to differences in refining procedures.

It is generally accepted (10, 16) that hydrogenation does not destroy tocopherols. Such is the case in the laboratory with tocopherols dissolved in a solvent, but Ward (17) has reported appreciable losses of tocopherols in oils under the conditions used during commercial hydrogenation and its attendant processes. Although we have no paired samples of an oil before and after hydrogenation, the tocopherol content of hydrogenated cottonseed oil and of partially hydrogenated safflower oil (obtained from margarine) is certainly at the lower limits of the range for unhydrogenated oils. The results with partially hydrogenated corn oil (from margarine) neither support nor refute a loss of tocopherol during commercial hydrogenation.

In our calculations of total tocopherols we have assumed that the tocopherol in the β- plus γ-tocopherol spot by paper chromatography (10) has been γ-tocopherol in all cases except wheat germ oil, in which it was assumed to be all β-tocopherol. This assumption is supported for most of the oils by other reports (see table 2 for refer-

[7] See footnote 3.
[8] See footnote 3.

TABLE 2

Literature values for tocopherol content of various vegetable oils

Oil	State	Total tocopherols	α-Tocopherol		Reference
		μg/g	μg/g	%	
Coconut	refined	0	0	—	(17)
	refined, hydrogenated	—	15	—	(18)
	crude	28	21	75	(19)
	unknown	83	36	43	(7)
Corn (maize)	refined	870	70	8	(7)
	crude	1167	47	4	(20)
	crude (original seed)	1157	58	5	(21)
	crude (172 days)	5647	2089	37	(21)
	crude (180 days)	2760	386	14	(21)
	crude (205 days)	1567	157	10	(21)
	unknown	910	100	11	(22)
	unknown	860	52	6	(23)
Cottonseed	refined	900	560	62	(7)
	refined	—	530	—	(24)
	crude	810	470	58	(17)
	unknown	—	325 [1]	—	(8)
	unknown	888	320 [1]	36	(9)
	unknown	810	470	58	(22)
	unknown	700	518	74	(23)
Linseed	crude	—	0	0	(25)
Mustard seed	unknown	525	84 [1]	16	(9)
	unknown	320	86	27	(22)
Olive	refined	72	72	100	(26)
	unknown	69	—	—	(7)
	unknown	144	117	81	(27)
	unknown	—	220	—	(28)
Peanut (groundnut)	refined	220	110	50	(7)
	refined	203	97	48	(17)
	crude	343	127	37	(19)
	unknown	195	70	36	(22)
	unknown	—	125 [2]	—	(28)
Rapeseed	unknown	—	225 [1]	—	(8)
	unknown	691	225 [1]	32	(9)
	unknown	560	151	27	(22)
Rice (germ)	crude	1828	1042	57	(27)
Rice (whole)	crude	—	—	47	(29)
Safflower seed	refined	—	816	—	(18)
Soybean	refined	1400	100	7	(7)
	refined	856	64	8	(17)
	unknown	—	110 [1]	—	(8)
	unknown	835	109 [1]	13	(9)
	unknown	1180	165	14	(22)
	unknown	830	124	15	(23)
	unknown	780	78	10	(27)
	unknown	1200	156	13	(30)
Sunflower seed	unknown	510	510	100	(23)
	unknown	597	454	76	(27)
	unknown	400	360	90	(30)
Wheat germ	refined	2300	2093 [1]	91	(9)
	crude	2667	1520	57	(20)
	crude	2900	1566	54	(31)
	crude	2800	1652	59	(32)
	crude	2853 [3]	1455 [3]	51	(33)
	unknown	—	2100 [1]	—	(8)
	unknown	2550	1428	56	(22)
	unknown	2620	1677	64	(23)
	unknown	—	—	49	(27)
	unknown	2600	1456	56	(30)

[1] Includes any zeta-tocopherol present. It is not known whether each pair of values reported by these authors (8, 9) actually represents duplicate analyses of a single sample.
[2] Average for 2 samples.
[3] Average for 3 samples.

ences), but little or no prior data are available for cocoa butter or for grapeseed, castor bean, linseed, and tung oils. δ-Tocopherol is a major constituent of tocopherols in soybean oil (20%) and castor bean oil (61%) and was observed in trace amounts in mustard seed oil and in one sample each of corn oil and safflower seed oil.

Paper chromatograms of the 6% eluates from rice bran oil and from one sample of coconut oil showed what was probably η-tocopherol, since Bro-Rasmussen and Hjarde (9) have shown that ε-tocopherol is eluted in the 4% fraction. We have not observed either ζ_1- or ζ_2-tocopherols in any of the samples reported above.

The α-tocopherol fractions from castor bean oil and from the samples of linseed oil contained unidentified reducing material(s) which moved with α-tocopherol in the first dimension of paper chromatography but which failed to move in the second dimension. The nature of this fraction is unknown; but calculated as α-tocopherol, it was equivalent to 115 μg/g in castor bean oil and 19 and 26 μg/g in the linseed oils.

Table 2 summarizes the data which other workers have obtained from some of these oils by using the above or closely related analytical procedures. Results obtained by a spectrophotometric method (19, 28) are also included, as are those obtained by Harris et al. (7) with older techniques.

Our values for total tocopherols were distinctly lower than those obtained by other workers for cottonseed, corn, rapeseed, safflower seed, sunflower seed, and wheat germ oils, but were about the same for the other oils. Our values for α-tocopherol varied from appreciably higher levels (e.g., corn oil) to appreciably lower levels (e.g., wheat germ oil). No comparisons are possible for the different rice fractions, for cocoa butter, or for castor bean, grapeseed, or tung oils. Unexpectedly, the values reported earlier by Harris et al. (7) were not inordinately high, although they were usually at the upper limit of the other values reported here.

We have mentioned above several of the factors which may influence the tocopherol content of oils. An obvious variable is the age of the oil and the stability of the tocopherols therein. We have not attempted to control this any more than would the average consumer. Table 3 illustrates with one plant, corn, some variations that de-

TABLE 3

Tocopherol content of various corn samples

Type of corn	Source	Lipid	Total tocopherols		α-Tocopherol		
		%	μg/g lipid	mg/454 g corn	μg/g lipid	mg/454 g corn	% of total tocopherols
Seed grain	Ohio-lot A	4.25	1421	27.4	296	5.7	21
Seed grain	Wisconsin	3.85	2499	43.6	549	9.6	22
Seed grain	Illinois	4.50	1487	30.3	440	9.0	30
Seed grain	Michigan	4.26	1356	26.2	334	6.5	25
Seed grain	Ohio-lot B	3.96	1792	32.3	398	7.2	22
Seed grain	Ohio-lot C	4.36	1474	29.2	367	7.3	25
Seed grain, moldy	Ohio-lot C	3.18	806	11.6	407	5.9	50
Seed grain	England [1]	8.40	1286	49.1	279	10.7	22
Soft seed on cob	England [1]	3.17	2269	32.7	281	4.1	12
Seed grain	Arizona	3.75	1708	29.1	476	8.1	28
Seed grain, ground, stored 3 months[2]	Arizona	3.82	1158	20.1	421	7.3	36
Seed grain, ground, stored 6 months[2]	Arizona	3.74	552	9.4	311	5.3	56

[1] Obtained through the courtesy of Dr. I. M. Sharman, Cambridge, England.
[2] Stored in cloth bag at room temperature (20–29°C).

pend on the history of the plant prior to processing.

The content of α- and total tocopherols varied over a twofold range among the samples from different sources. The samples from England suggested a dependence of content on time of harvest, confirming the striking changes noted by Green (21) during the maturation of maize and other plants. Significant losses of tocopherols occurred in grain which became moldy or which was ground and stored for 6 months. In both cases, the non-α-tocopherols declined much more rapidly than α-tocopherol, suggesting an antioxidant protection of α-tocopherol by the other tocopherols.

The data on corn oils in tables 1 and 3 indicate appreciable losses of tocopherols during refining, although an enrichment may take place in the relative amount of α-tocopherol. On the other hand, corn appears to be a better source of α-tocopherol in animal feeds than has been thought hitherto. The values in table 3 show that the percentage of α-tocopherol and the content of total tocopherols are each about twice previous estimates (12). The combination of these 2 factors gives an α-tocopherol content for corn approximately 4 times the content reported in the older literature.

One explanation for this difference may be the observation of Hivon and Quackenbush (34) that corn contains unidentified materials which inhibit the analysis of tocopherols with 2,2′-bipyridine and $FeCl_3$ reagents. Removal of these substances prior to analysis of crude corn lipids gave an apparent total tocopherol content of 500 to 2830 μg/g oil. This range encompasses the range of our values.

The significance of these observations in relation to increased intakes of polyunsaturated fatty acids by man and other animals cannot yet be evaluated definitively. In a current assessment of vitamin E nutrition in man, Harris and Embree (35) examine available information on tocopherol:fatty acid relationships and on the content of these materials in the average diet in the United States. They estimate that the requirement of man is 0.6 mg of *d*-α-tocopherol/g of polyunsaturated fatty acid.

The question can then be raised concerning the possible effect on vitamin E nutrition of a substantial *increase* in dietary polyunsaturated fatty acid via an increased consumption of vegetable oil. Vitamin E occurs naturally as *d*-α-tocopherol, and, for most common vegetable oils, increased consumption of α-tocopherol automatically accompanies increased consumption of polyunsaturated fatty acids. Our *average* values for refined, unhydrogenated oils show that cottonseed, corn, safflower, and soybean oils contain 0.31, 0.19, 0.31, and 0.16 mg, respectively, of *d*-α-tocopherol/g of oil. Since these oils contain approximately 50, 56, 72, and 60%, respectively, of polyunsaturated fatty acids (36), they supply about 0.65, 0.36, 0.45, and 0.28 mg, respectively, of *d*-α-tocopherol/g of polyunsaturated fatty acid. Only in cottonseed oil is protection inherent (near the estimated requirement of 0.6 mg *d*-α-tocopherol/g of polyunsaturated fatty acid).

The over-all balance of α-tocopherol and polyunsaturated acids in a diet will obviously be determined by all dietary components. If refined vegetable oils such as corn, safflower, and soybean contain insufficient vitamin E to counterbalance the effect of their polyunsaturated fatty acids, the deficit must be supplied either by other dietary components or by body reserves. The possibility cannot be ignored that an appreciable increase in consumption of such oils, whether as supplemental or replacement fat, could result in gradual depletion and eventual deficiency of vitamin E.

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Amino Acids in Dog Blood and Gut Contents after Feeding Zein[1]

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ABSTRACT Two hours after feeding zein the animals were anesthetized and the abdomen opened. Blood was drawn simultaneously from the carotid artery and mesenteric, portal and jugular veins. Contents were removed from the portion of jejunum drained by the intubated mesenteric vein. Amino acid concentrations increased above fasting values but generally the increases were greatest in the mesenteric vein. Molar ratios of amino acids in zein were different from both gut contents and blood plasma. Lysine and tryptophan, which are virtually absent from zein, were present in gut contents and there was no depression of concentration of these two amino acids in mesenteric blood.

Ingested labeled protein is mixed with several times its mass of endogenous protein in the lumen of the small intestine (1). This explains an earlier observation that the molar ratios of amino acids in the gut lumen are not greatly altered by the type of test meal ingested (2). Feeding lean beef results in an increase in total blood plasma amino acids, especially in mesenteric and portal vein blood, but the molar ratios are not what might be expected from the amino acid composition of lean beef (3).

The work reported below was designed to provide more information with respect to the role of the digestive tract in the homeostasis of blood plasma amino acids as revealed by simultaneous sampling of blood and gut contents.

METHODS

Five dogs (15- to 20-kg body weight) were fed the standard animal house dog food[3] for 2 weeks or longer before being used for experiment. After an 18-hour fast, blood was drawn from the saphenous vein. Immediately thereafter the animals, with some coaxing, were fed 45 g of zein (isonitrogenous with 200 g of lean beef fed in previous experiments (3)) made into a paste with water. They drank water at will. The dogs were anesthetized with pentobarbital sodium 1.5 hours after feeding and the abdomen opened. Polyethylene tubing was threaded through a tributary into the portal vein and the tip of the tube placed as close as possible to the liver. A mesenteric vein, draining a portion of the jejunum about 50 cm caudad from the ligament of Treitz, was intubated. Two hours after feeding, blood was drawn simultaneously from the carotid artery and jugular, portal and mesenteric veins. All blood samples were heparinized and centrifuged. The contents of that portion of the jejunum drained by the intubated mesenteric vein were collected by washing out the lumen 3 times with distilled water. Contents and washings were combined and quickly lyophilized.

Blood plasma and gut contents were analyzed for amino acids (4). Total N was determined by micro-Kjeldahl, and NPN by nesslerization (5).

RESULTS

The results of amino acid analyses are assembled in table 1. Gut contents were recovered as a slurry, making volume measurement meaningless, and therefore amino acid concentrartion was related to the total NPN in the contents. Total micromoles of amino acid are simply the sum of the means in each column in table 1.

With the exception of lysine, the average concentration of each amino acid is greater in mesenteric vein blood than in portal vein blood. Some of the differences

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[3] "Play-Pup," manufactured by Nutrition Associates, Inc., Orchard Park, New York.

TABLE 1

Amino acids in dog blood and gut contents

Amino acid	Fasting	2 Hours after feeding zein				
	Saphenous vein	Jugular vein	Carotid artery	Mesenteric vein	Portal vein	Gut contents[1]
	μmoles/100 ml blood plasma					
Arginine	4.4 ± 0.6[2](4)[3]	4.7 ± 0.5(4)	3.1 ± 1.6(3)	4.7 ± 0.9(3)	4.0 ± 1.9(4)	26.2 ± 5.3(3)
Histidine	4.9 ± 1.3 (5)	7.5 ± 1.1(5)	5.4 ± 1.9(4)	4.4 ± 1.5(4)	4.5 ± 1.8(4)	15.9 ± 7.2(5)
Isoleucine	5.8 ± 0.8 (5)	7.2 ± 0.8(5)	7.9 ± 0.7(4)	7.2 ± 0.7(5)	6.0 ± 1.5(4)	23.2 ± 4.6(5)
Leucine	9.6 ± 0.7 (5)	22.3 ± 3.7(5)	23.2 ± 4.2(5)	27.6 ± 4.7(5)	22.8 ± 5.6(4)	56.8 ± 11.2(5)
Lysine	11.3 ± 2.6 (5)	14.2 ± 2.3(5)	11.1 ± 1.3(4)	9.2 ± 3.7(4)	11.9 ± 2.1(4)	27.5 ± 7.6(5)
Methionine	3.5 ± 0.2 (5)	4.4 ± 0.8(5)	3.8 ± 0.4(4)	4.1 ± 0.5(5)	3.5 ± 0.6(4)	7.2 ± 1.8(5)
Phenylalanine	5.1 ± 0.5 (5)	7.9 ± 1.2(3)	7.3 ± 0.3(3)	8.1 ± 1.2(5)	4.9 ± 0.5(4)	24.5 ± 5.1(5)
Threonine	24.3 ± 5.8 (4)	33.1 ± 6.8(3)	18.0 ± 6.0(4)	26.8 ± 5.3(4)	20.5 ± 5.5(3)	24.0 ± 3.8(5)
Tryptophan	0.9 ± 0.6 (5)	2.2 ± 0.9(5)	2.8 ± 1.8(3)	1.1 ± 0.7(4)	0.9 ± 0.9(4)	2.0 ± 0.4(5)
Valine	15.0 ± 1.3 (5)	17.4 ± 0.9(5)	16.1 ± 1.0(5)	20.4 ± 2.8(5)	14.4 ± 2.1(4)	33.4 ± 6.3(5)
Alanine	41.6 ± 4.0 (5)	58.1 ± 5.0(5)	43.5 ± 6.8(5)	65.6 ± 7.9(5)	48.4 ± 6.6(4)	53.3 ± 6.9(5)
Aspartic acid	1.2 ± 0.3 (4)	1.8 ± 0.4(4)	1.1 ± 0.5(4)	1.5 ± 0.2(4)	1.2 ± 0.3(3)	15.9 ± 2.6(5)
Glutamic acid	4.4 ± 0.7 (5)	9.3 ± 1.1(5)	5.0 ± 1.4(5)	10.3 ± 2.4(5)	5.8 ± 1.4(4)	34.7 ± 3.7(5)
Glycine	17.9 ± 2.8 (5)	25.5 ± 4.4(5)	18.8 ± 6.7(5)	19.3 ± 1.8(5)	14.2 ± 1.0(4)	29.4 ± 5.1(5)
Proline	9.7 ± 2.7 (4)	19.2 ± 2.0(5)	15.8 ± 3.1(5)	25.5 ± 3.4(5)	18.0 ± 5.3(4)	24.7 ± 1.9(5)
Serine	15.0 ± 2.2 (4)	20.3 ± 1.8(4)	15.5 ± 3.3(3)	18.2 ± 2.2(4)	13.7 ± 1.7(3)	32.7 ± 5.3(5)
Tyrosine	3.9 ± 0.5 (5)	5.5 ± 1.0(3)	5.7 ± 0.9(4)	6.8 ± 1.3(5)	2.7 ± 1.0(4)	24.1 ± 1.2(4)
Total, μmoles	179	274	223	267	195	456

[1] Micromoles/30 mg NPN (approximate NPN/100 ml plasma).
[2] Mean ± SE.
[3] Number of experiments indicated in parentheses.

are small and only one of them is significant (tyrosine; $P = 0.03$). Amino acid concentrations in postprandial bloods from the carotid artery and the mesenteric and jugular veins are generally similar.

Amino acid concentrations are lower in peripheral venous blood after an 18-hour fast (saphenous) than 2 hours after feeding (jugular). The bloods came from the same animal but the fasting sample was taken without anesthesia and from a different peripheral vein. In the conscious, untrained dog it is more convenient to draw blood from the saphenous vein than from the jugular. Although fasting values are all lower, only the following differences are significant: leucine ($P = 0.01$); phenylalanine ($P = 0.05$); alanine ($P = 0.03$); glutamic acid ($P = 0.01$); proline ($P = 0.02$).

Comparisons of the amino acid mixtures in blood plasma, gut contents and ingested protein are facilitated by the use of molar ratios as given in table 2. In these experiments isoleucine fluctuated less than any of the other indispensable amino acids and all molar ratios are based on isoleucine concentration as unity.

COMMENT

These results provide further evidence that, in a single feeding, the amino acid composition of ingested protein does not greatly affect the amino acid mixture existing in the jejunum during digestion. As indicated in table 2, the molar ratios of amino acids in zein, the ingested protein, are generally different from those observed in gut contents. There is better agreement between the amino acid mixtures found in gut contents and the mesenteric vein. Leucine and aspartic acid are exceptions that are partially explained by the work of Neame and Wiseman (6). They demonstrated that glutamic and aspartic acids, as they pass through the mucosa, participate in transamination reactions which result in a reduction of the concentrations of these amino acids and an increase in alanine in mesenteric blood. Zein is virtually devoid of tryptophan and lysine. If the amino acid composition of ingested protein were immediately crucial in determining the amino acid composition of the blood flowing from the small intestine, deficiencies of this sort should be reflected in mesenteric blood. It is evident from these and other data from this laboratory that the amino acid pattern in blood plasma is not subject to great variation, within 2 hours of feeding, regardless of the type of protein ingested.

Several factors may be involved in determining the concentration of amino

TABLE 2

Molar ratios of amino acids in blood, gut contents and test meal protein (zein) (isoleucine = 1.00)

Amino acid	Fasting	2 Hours after feeding zein					Test meal protein,[1] zein
	Saphenous vein	Jugular vein	Carotid artery	Mesenteric vein	Portal vein	Gut contents	
Arginine	0.73	0.65	0.38	0.65	0.67	1.13	0.17
Histidine	0.84	1.04	0.68	0.61	0.75	0.69	0.14
Isoleucine	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Leucine	1.65	3.10	2.94	3.84	3.80	2.45	3.30
Lysine	1.95	1.97	1.41	1.28	1.98	1.18	0.01
Methionine	0.60	0.61	0.48	0.57	0.59	0.31	0.25
Phenylalanine	0.88	1.10	0.92	1.12	0.82	1.06	0.70
Threonine	4.20	4.60	2.28	3.72	3.42	1.03	0.41
Tryptophan	0.16	0.36	0.35	0.15	0.15	0.09	0.01
Valine	2.59	2.62	2.03	2.83	2.40	1.44	0.40
Alanine	7.18	8.07	5.50	9.12	8.07	2.30	0.22
Aspartic acid	0.27	0.25	0.14	0.21	0.20	0.69	0.45
Glutamic acid	0.73	1.29	0.63	1.43	0.97	1.49	3.47
Glycine	3.09	3.54	2.38	2.68	2.37	1.27	0.10
Proline	1.67	2.67	2.00	3.54	3.00	1.06	1.56
Serine	2.59	2.82	1.96	2.53	2.28	1.41	1.32
Tyrosine	0.67	0.76	0.72	0.95	0.45	1.04	0.50

[1] Computed from data in Amino Acid Handbook, eds., R. J. Block and K. W. Weiss. 1956. Charles C Thomas, Springfield, Illinois.

acids in mesenteric vein blood. The mesenteric artery delivers its mixture of amino acids to the small intestine which, to meet its own anabolic demands, may remove some amino acids from the capillary circulation before the blood enters the mesenteric vein. There is doubtless, also, a catabolic supply of amino acids which could enter the capillary blood. The mixture of free amino acids present in the lumen of the gut and available for absorption is determined by the admixture of exogenous (food) proteins and endogenous proteins derived from digestive secretions and sloughed mucosa (1, 2, 7). Presumably amino acids pass through the mucosal cells in the process of absorption and therefore it seems probable that some of them are incorporated directely into cellular proteins without entering the vascular system. The concentrations of absorbed amino acids that finally enter the mesenteric vein might differ appreciably from those in the gut lumen, owing to the amino acid metabolism of the absorptive cells themselves.

The amino acid concentration in portal vein blood is a resultant of the mixing of venous blood from the small gut and other abdominal viscera and it could change with a change in protein metabolism of any of the organs concerned. If the pancreas, for example, were actively synthesizing enzymes to replace those that had been discharged in pancreatic juice, the amino acid mixture in the pancreatico-duodenal vein could be different from the mixture in fasting or the post-absorptive state.

Longenecker and Hause (8) fed casein, gelatin and wheat gluten to dogs and sampled heart blood at hourly intervals. Plasma amino acids increased as early as 1 hour after feeding, but there was no consistent correlation between the amino acid pattern of plasma and that of the protein ingested. On realimentation of the fasting rat, the small gut repletes its protein stores most rapidly, with the liver a close second (9, 10). If this happens in the dog early in the postprandial period, Longenecker and Hause may have been dealing with plasma amino acid changes due primarily to the protein metabolism of the gut and liver. It seems unjustified to assume, as they did, that indispensable amino acids, in acute experiments, are removed from plasma in proportion to the dog's long-term requirements, which are probably not yet firmly established.

The molar ratios shown in table 2 show very little resemblance between zein, the ingested protein, and either the gut contents or mesenteric blood. The most striking differences, as expected, are in the ratios of lysine and tryptophan, but arginine, histidine, threonine and valine also show large differences.

The present work confirms the idea that the mixing of exogenous and endogenous proteins in the gut serves as a homeostatic mechanism to prevent large changes in molar ratios of the free amino acids in gut contents (1, 2). Under these conditions a single feeding of any protein, regardless of its amino acid composition, should not be expected to cause an appreciable change in the mixture of amino acids available for absorption. If an incomplete protein were fed as part of the ration for some time, it is obvious that an indispensable amino acid deficiency would develop and the normal homeostasis would fail.

Guggenheim et al. (11) reported that lysine and methionine concentration in portal blood of the rat after feeding did not correlate closely with the concentration of the amino acids in test meal proteins. Feeding starch caused just as great an increase in lysine as observed after feeding wheat gluten or zein. Zein and starch are devoid of lysine but the amount of endogenous protein in the lumen of the gut during digestion could provide this increase in lysine (1).

Dietary zein fed for 1 to 4 weeks to chicks resulted in a lowered plasma lysine concentration (12). Tryptophan was not affected and threonine and leucine were increased. In protein-depleted rats an amino acid imbalance (low in histidine) caused a decrease in plasma histidine in 2 days (13). There was a simultaneous increase in lysine, threonine and phenylalanine. Rats maintained with 6% fibrin or protein-free diets for a few days were fed a diet yielding an imbalance of amino acids and the intake of food began to diminish within a few hours (14).

The fact that rats may reduce their intake of a diet deficient in an indispensable amino acid or yielding an imbalanced amino acid mixture within hours after it is offered to them (15), suggests that certain signals may reach the satiety center in the hypothalamus even before much of the diet has been absorbed and before the amino acid composition of plasma has been appreciably changed. Electrical activity in afferent mesenteric nerves is increased by irrigating a loop of jejunum with glucose or amino acid solutions (16), suggesting the presence of chemoreceptors in the mucosa which may play a role in the regulation of food intake before a change in chemical composition of the blood initiates regulatory changes in the central nervous system.

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Factors Influencing the Induction of Fatty Liver by Orotic Acid[1,2]

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ABSTRACT Female Wistar strain rats were fed purified diets containing orotic acid, and the influence of diet on orotic acid induced fatty liver was studied. Starved animals receiving daily 100 to 200 mg orotic acid or other pyrimidines or purines for 1 to 3 days did not develop fatty livers. Animals fed ad libitum the purified diet and tube-fed separately 100 mg orotic acid daily for 3 days did not develop fatty livers. Animals force-fed for 3 days an adequate amount of purified diet containing 1% orotic acid but not those force-fed one-half amount of diet containing 2% orotic acid developed fatty livers. Animals force-fed for 3 days an orotic acid-containing diet in which casein was replaced by casein hydrolyzate or purified amino acids failed to develop fatty livers. Animals fed ad libitum for 7 days an orotic acid-containing diet, in which casein, lactalbumin or soybean (protein or its hydrolyzate) was used, developed marked fatty livers in all animals except those fed the casein hydrolyzate diet where only mild fatty livers developed. These results indicate the importance of the nature and quantity of the diet in the induction of fatty liver by orotic acid.

Orotic acid (6-carboxy-2,4-dihydroxypyrimidine), a normal component of milk, is an intermediate in pyrimidine nucleotide synthesis in microorganisms and in higher animals. It has been reported that rats fed purified diets containing orotic acid develop fatty livers with a periportal distribution of the lipid (1, 2). The addition of adenine to these diets prevents or reverses the fatty liver (2). The hepatic lipid accumulation is more severe in female than in male rats (3). During the study on the sex difference, it was observed that the quantity and composition of the diet had a striking influence upon the rate and degree of development of the fatty liver. These effects have been studied in some detail and form the subject of this communication.

METHODS

Female rats of the Wistar strain[3] weighing from 165 to 220 g were used. The animals were maintained with a commercial chow[4] and were fasted overnight before beginning the special diets. All animals had free access to water. In all experiments, several groups of rats of the same age and weight were used. The animals were housed in individual wire cages in an air conditioned room maintained at 25.5°C.

The basal diet was similar to that used in an earlier study (3) and was composed of 16% protein or amino acids, 5% corn oil,[5] 4% salts (4), 5% vitamin-sucrose mixture (5), and 70% sucrose. In most experiments vitamin free casein[6] was the source of protein, but in some, lactalbumin[7] or soybean[8] was used. In addition, casein hydrolyzate[9] supplemented with 0.2% DL-tryptophan, lactalbumin hydrolyzate,[10] soybean hydrolyzate,[11] or purified amino acids were substituted for protein in some experiments. The essential and nonessential amino acid mixture was that described in an earlier report (6). In one

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[3] Carworth Farms, New City, New York.

[4] Wayne Lab-Blox, Allied Mills Inc., Chicago, Illinois.

[5] Mazola, Corn Products Company, New York.

[6] "Vitamin Free" Casein, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[7] Lactalbumin, Nutritional Biochemicals Corporation, Cleveland, Ohio.

[8] Promine, Central Soya Company, Inc., Chicago, Illinois.

[9] "Vitamin Free" Casein Hydrolysate (Acid), Nutrition Biochemicals Corporation, Cleveland, Ohio.

[10] Lactalbumin Hydrolysate (Enzymatic), Nutritional Biochemicals Corporation, Cleveland, Ohio.

[11] Soybean Hydrolysate (Enzymatic), Nutritional Biochemicals Corporation, Cleveland, Ohio.

experiment the complete amino acid diet used previously (6) was substituted for the basal diet. Orotic acid was added to the experimental groups at a level of 1% in place of a corresponding amount of sucrose.

In the force-feeding experiments rats were tube-fed according to the method of Shay and Gruenstein (7) using plastic tubes. The basal ration was force-fed to the control rats throughout and to all experimental animals for 2 days prior to beginning the experimental orotic acid-containing diets. Each ration was blended with distilled water so that one milliliter of diet mixture contained 0.7 g of diet. The diet mixtures were tube-fed 3 times daily at 8 AM, 1 PM, and 5 PM for 3 days and each animal received an average daily feeding of 0.6 g of ration/10 g of initial body weight.

In 2 force-feeding experiments of 3 days duration, a radioactive amino acid was injected intraperitoneally into rats 3 hours before killing. In one experiment, 0.037 mg (1.66 μc) of L-leucine monohydrochloride biologically labeled with C^{14}, prepared as an aqueous solution, was administered. In another experiment, 0.077 mg (1.66 μc) of DL-phenylalanine-3-C^{14} hydrochloride was administered. Aliquots of aqueous liver homogenate were precipitated with 10% trichloroacetic acid (TCA) and were subsequently washed 4 times with 5% TCA and once each with 95% ethanol, ethanol-ethyl ether mixture (3:1), and ethyl ether. Ten milligrams of the corresponding nonradioactive amino acid were added to the third TCA wash. The dried protein powder was plated on Whatman no. 540 filter paper and counted in windowless flow counter. The quantity of protein in the counted sample was weighed.

In one experiment radioactive 6-C^{14}-orotic acid was added to the casein and casein hydrolyzate diets (1 μc in 5 ml diet). These diet mixtures were tube-fed to 2 groups of animals that were killed after 1, 3 and 5 hours and total radioactivity of liver, blood, and gastrointestinal tract was measured.

In the ad libitum experiments the rats had free access to the dry diet mixture for 3 to 7 days. Diet was available until the morning the animals were killed. Rats were weighed at the beginning and at the termination of each experiment. The liver was removed rapidly and weighed, and a tared aliquot of approximately 1 g was frozen for subsequent lipid analysis. Liver total lipids were extracted according to the method of Folch et al. (8) and were determined gravimetrically after evaporation of the solvent under a stream of N_2 to constant weight. Protein was determined by the biuret method, according to Layne (9).

RESULTS

Acute toxicity studies. In previous work, orotic acid was administered to animals as a dietary component and not by itself in the absence of food. Since it is important to determine whether orotic acid as such has any toxic effect, a series of experiments were performed to test this. The effects of orotic acid upon the liver of the starved rat are indicated in table 1. The orotic acid was force-fed as an aqueous suspension, instead of being administered parenterally, because of its high insolubility in water. In addition we studied the influence of other pyrimidine and purine compounds given intraperitoneally or by tube feeding to starved rats (table 1). Fatty livers did not develop in animals receiving orotic acid, uridine, cytidine, cytosine, uracil, xanthine, or guanine. These results indicate that orotic acid by itself under these conditions does not induce a fatty liver and suggests the existence of a more complex interaction between the diet and the pyrimidine in the pathogenesis of the fatty liver.

In the next group of experiments, we investigated whether orotic acid would induce a fatty liver in fed rats when administered separately by stomach tube. Table 2 summarizes the results of these experiments. Rats fed ad libitum the basal 16% casein purified diet containing 1% orotic acid developed fatty liver in 3 days. On the other hand, when rats were fed ad libitum the same diet without orotic acid but received orotic acid separately 3 times daily by stomach tube, they did not develop fatty liver. Similarly when rats were fed ad libitum a high carbohydrate, high protein or high fat diet with orotic acid tube-fed separately, they did not develop fatty livers. These results indicate that orotic

TABLE 1

Acute toxicity studies on starved rats

Agent	Dose	Route of administration[1]	Duration	No. of rats	Liver weight	Liver lipid	
	mg/day		*days*		*g/100 g body wt*	*%*	*mg/100 g body wt*
Orotic acid	100	FF	1	7	2.93 ± 0.09[2]	5.9 ± 0.2[2]	170 ± 12[2]
Orotic acid	100	FF	3	7	3.13 ± 0.11	4.6 ± 0.1	144 ± 6
Uridine	100	FF	1	5	2.92 ± 0.10	5.9 ± 0.3	170 ± 8
Uridine	100	ip	1	10	2.99 ± 0.06	5.6 ± 0.2	170 ± 9
Uridine	200	ip	1	5	3.05 ± 0.07	5.1 ± 0.1	155 ± 6
Cytidine	200	ip	1	4	2.55 ± 0.09	6.3 ± 0.3	162 ± 12
Cytosine	200	ip	1	4	2.51 ± 0.05	6.8 ± 0.7	170 ± 13
Uracil	200	ip	1	3	2.91 ± 0.09	7.0 ± 0.6	202 ± 9
Xanthine	200	ip	1	3	2.76 ± 0.25	5.8 ± 0.6	160 ± 18
Guanine	200	ip	1	4	2.53 ± 0.05	6.0 ± 0.2	152 ± 8
Control	0	ip	1	5	3.05 ± 0.04	4.9 ± 0.3	150 ± 7

[1] FF = force-fed; ip = intraperitoneal.
[2] Mean value ± SE of mean.

TABLE 2

Liver lipid of rats fed diets ad libitum for 3 days

Diet		Orotic acid, 100 mg/day		No. of rats	Liver weight	Liver lipid	
Type[1]	Amount consumed	Ad libitum	Force-fed				
	g/day				*g/100 g body wt*	*%*	*mg/100 g body wt*
Casein, 16%	11.5	+		6	5.71 ± 0.32[2]	11.3 ± 0.9[2]	640 ± 40[2]
Casein, 16%	12.1		+	14	4.66 ± 0.13[3]	5.9 ± 0.5[3]	277 ± 31[3]
High carbohydrate	8.1		+	7	4.54 ± 0.32[4]	5.4 ± 0.5[3]	251 ± 40[3]
High protein	7.6		+	7	4.43 ± 0.25[3]	3.3 ± 0.1[3]	147 ± 8[3]
High fat	5.1		+	7	3.39 ± 0.21[3]	8.6 ± 0.9[5]	296 ± 41[3]

[1] High carbohydrate diet (91% sucrose, 5% vitamin-sucrose mixture, 4% salts).
High protein diet (91% casein, 5% vitamin-sucrose mixture, 4% salts).
High fat diet (91% corn oil, 5% vitamin-sucrose mixture, 4% salts).
[2] Mean value ± SE of mean.
[3] $P < 0.01$ (highly significant). All groups compared with group on 16% casein and orotic acid ad libitum.
[4] P between 0.01 and 0.05 (probably significant).
[5] $P > 0.05$ (not significant).

acid must be given together with the basal purified diet to induce fatty liver.

Next an experiment was performed to observe the influence of the quantity of purified diet consumed on the fatty liver induction. Rats were force-fed for 3 days 2 different amounts of the 16% basal casein diet containing orotic acid. The daily orotic acid intake was kept constant. Table 3 summarizes these results. Rats fed 10.6 g diet/day developed fatty livers, whereas rats fed one-half amounts of diet (5.3 g diet/day) did not.

To study the influence of the level of dietary casein on orotic acid-induced fatty liver, rats were force-fed for 3 days diets containing from zero to 32% casein with and without orotic acid. Sucrose was substituted for the casein to keep the diets isocaloric. It is apparent from table 4 that casein (4 to 32%) in the diet is necessary for the induction of fatty liver due to orotic acid. Rats fed the diets containing orotic acid but devoid of casein with either high carbohydrate or high fat failed to develop fatty livers. It is noteworthy that increased amounts of lipid appeared in the livers of the 2 control groups fed low protein (8% and especially 4% casein) without orotic acid, results observed previously in rats force-fed low protein diets for 3 days (10).

After learning that casein was essential in the diet for the induction of fatty liver due to orotic acid, it became pertinent to determine whether amino acids would have the same effect if substituted for casein.

TABLE 3

Liver lipid of rats force-fed diet containing 1% orotic acid for 3 days

Diet	Daily intake		No. of rats	Liver weight	Liver lipid	
	Diet	Orotic acid				
	g	*mg*		*g/100 g body wt*	*%*	*mg/100 g body wt*
Casein, 16%	10.6	106	3	4.19 ± 0.23[1]	10.2 ± 1.8[1]	433 ± 32[1]
Casein, 16%	5.3	106	8	3.49 ± 0.12[2]	4.5 ± 0.3[2]	157 ± 6[3]

[1] Mean value ± SE of mean.
[2] P between 0.01 and 0.05 (probably significant).
[3] P < 0.01 (highly significant).

TABLE 4

Liver lipid of rats force-fed diets containing zero to 32% casein and 1% orotic acid for 3 days

Diet[1]	Orotic acid, 110 mg/day	No. of rats	Liver weight	Liver lipid	
			g/100 g body wt	*%*	*mg/100 g body wt*
Casein, 32%	—	4	3.87 ± 0.25[2]	7.4 ± 1.2[2]	294 ± 60[2]
	+	10	4.87 ± 0.15[3]	10.5 ± 1.0[5]	527 ± 69[4]
Casein, 16%	—	16	3.63 ± 0.10	8.0 ± 0.5	293 ± 21
	+	21	4.79 ± 0.11[3]	12.8 ± 0.3[3]	626 ± 56[3]
Casein, 8%	—	4	3.82 ± 0.16	9.7 ± 1.0	376 ± 50
	+	8	4.98 ± 0.15[3]	13.3 ± 1.1[4]	658 ± 60[3]
Casein, 4%	—	3	3.60 ± 0.16	11.9 ± 1.0	429 ± 49
	+	8	5.02 ± 0.14[3]	12.3 ± 1.3[5]	620 ± 68[4]
No casein, high carbohydrate	+	4	3.82 ± 0.08	6.6 ± 0.8	253 ± 34
No casein, high fat	+	5	3.44 ± 0.19	5.5 ± 0.2	190 ± 10

[1] All diets were isocaloric.
No casein — high carbohydrate diet contained no casein, no fat, vitamin-sucrose mixture, salts and high sucrose plus dextrin.
No casein — high fat diet contained no casein, no carbohydrate, vitamin-sucrose mixture, salts and high corn oil.
[2] Mean value ± SE of mean.
[3] P < 0.01 (highly significant).
[4] P between 0.01 and 0.05 (probably significant).
[5] P > 0.05 (not significant).

In most of these experiments casein hydrolyzate plus added tryptophan or purified amino acids were used in place of casein. In one experiment a different amino acid diet (6) was used. Rats were force-fed for 3 days these diets with or without orotic acid. The results of these experiments are presented in table 5. In contrast with the rats force-fed casein plus orotic acid, the animals fed casein hydrolyzate or amino acids plus orotic acid did not develop fatty livers. Rats force-fed the amino acid diet plus orotic acid had a small increase in total liver lipid over those on the amino acid diet without orotic acid. However, these liver lipid levels were low in comparison with those of the other groups.

In an attempt to learn what might be responsible for the different response of the rat when fed orotic acid with casein or with casein hydrolyzate, 2 experiments were performed. In the first experiment, the absorption of orotic acid from the gastrointestinal tract was measured when it was fed with the intact protein and with the protein hydrolyzate. Radioactive 6-C^{14}-orotic acid, 1 μc in 5 ml diet (casein or casein hydrolyzate) was tube-fed to 2 groups of rats. Control animals received 1 μc in 5 ml distilled water. Two rats of

TABLE 5

Liver lipid of rats force-fed diets containing 1% orotic acid for 3 days

Diet[1]	Orotic acid, 110 mg/day	No. of rats	Liver weight	Liver lipid	
			g/100 g body wt	*%*	*mg/100 g body wt*
Casein	—	16	3.63 ± 0.10[2]	8.0 ± 0.5[2]	293 ± 21[2]
	+	21	4.79 ± 0.11[3]	12.8 ± 0.3[3]	626 ± 56[3]
Casein hydrolyzate acid	—	4	4.00 ± 0.26	7.9 ± 1.0	321 ± 62
	+	9	4.57 ± 0.20[4]	6.8 ± 0.8[4]	312 ± 46[4]
Amino acids	—	5	3.49 ± 0.10	7.0 ± 0.9	236 ± 35
	+	3	3.86 ± 0.16[4]	4.6 ± 0.3[5]	179 ± 17[4]
Amino acid diet	—	5	3.16 ± 0.05	4.6 ± 0.3	144 ± 12
	+	4	4.10 ± 0.02[3]	4.9 ± 0.4[4]	202 ± 3[3]

[1] Casein diet (16% "Vitamin Free" Casein, Nutritional Biochemicals Corp., Cleveland, Ohio).
Casein hydrolyzate-acid diet (15.8% "Vitamin Free" Casein Hydrolysate (Acid), Nutritional Biochemicals Corp., + 0.2% DL-tryptophan).
Amino acids (10.5% essential amino acids and 8.1% non-essential amino acids (6)).
Amino acid diet (purified diet used in other studies (6)).

[2] Mean value ± SE of mean.

[3] $P < 0.01$ (highly significant).

[4] $P > 0.05$ (not significant).

[5] P between 0.01 and 0.05 (probably significant).

each group were then killed after 1, 3 and 5 hours and total radioactivity of liver, blood and gastrointestinal tract (esophagus to anus with contents) was measured. Since the radioactivity in the blood and liver of animals of each group were similar at each time interval, the results have been combined and are expressed as the mean for all 3 time intervals. The total radioactivity in the blood, liver and gastrointestinal tract of the 3 groups are respectively as follows: casein, 970, 6,970 and 50,200; casein hydrolyzate, 790, 5,460 and 41,800; and water, 2,210, 15,420 and 44,500. The values in each group are the mean of 6 animals and are expressed as counts per minute per organ or per 100 ml blood. The results indicate only minor differences, the largest ones being a decrease of 21 and 19% for liver and blood, respectively, in rats fed the casein hydrolyzate diet in comparison with those fed the casein diet. Since the radioactivity remaining in the gastrointestinal tract was lower (17%) in rats fed the casein hydrolyzate diet than in rats fed the casein diet, the overall absorption from the gastrointestinal tract was probably as great or greater in rats fed the casein hydrolyzate diet than in those fed the casein diet. These results suggest that orotic acid absorption is similar with both diets.

In the second experiment, an attempt was made to compensate for the more rapid absorption of amino acids from the gastrointestinal tract when the amino acids are given free, as in casein hydrolyzate, rather than as protein. Two groups of animals were force-fed the casein hydrolyzate diet for 3 days; one group was fed the diet containing 1% orotic acid, and the second group was tube-fed orotic acid alone 2 hours before each feeding of casein hydrolyzate diet. The daily orotic acid intake was 119 mg for the first group and 100 mg for the second group. For comparison, animals of 2 additional groups were force-fed the casein diet with or without the added orotic acid. The results summarized in table 6 indicate that the earlier administration of orotic had no influence on the degree of fatty liver due to casein hydrolyzate plus orotic acid. Animals force-fed the casein diet containing orotic acid developed a marked degree of fatty change of the liver which was greater than similarly treated animals described in table 3. The difference is probably related to the higher diet and orotic acid intake (12%) in the present over that in the former experiment. The results of the preceding 2 experiments suggest that gastrointestinal absorption of orotic acid or of amino acids in animals fed the casein hydrolyzate diet is

TABLE 6

Liver lipid of rats force-fed casein hydrolyzate diet or casein diet and orotic acid for 3 days

Diet	Orotic acid	No. of rats	Liver weight	Liver lipid	
	mg/day		*g/100 g body wt*	*%*	*mg/100 g body wt*
Casein hydrolyzate	119	4	5.13 ± 0.15[1]	8.5 ± 1.2[1]	433 ± 57[1]
Casein hydrolyzate	100[2]	3	5.98 ± 0.38	7.5 ± 1.0	454 ± 85
Casein	119	4	5.40 ± 0.10	16.2 ± 1.9	878 ± 103
Casein	0	4	4.08 ± 0.11	9.4 ± 1.0	383 ± 18

[1] Mean value ± SE of mean.
[2] Orotic acid was tube-fed 2 hours before each diet feeding.

TABLE 7

Liver lipid of rats fed ad libitum diets containing 1% orotic acid for 1 week

Diet	Orotic acid	No. of rats	Liver weight	Liver lipid	
	mg/day		*g/100 g body wt*	*%*	*mg/100 g body wt*
Casein	0	7	4.35 ± 0.12[2]	6.3 ± 0.5[2]	273 ± 16[2]
	126	7	5.43 ± 0.28[3]	18.7 ± 0.8[3]	1054 ± 76[3]
Casein hydrolyzate	0	4	4.26 ± 0.08	7.6 ± 0.1	321 ± 22
	131	5	5.12 ± 0.19[3]	10.8 ± 2.3[4]	551 ± 111[4]
Soybean	0	4	3.81 ± 0.23	4.6 ± 0.1	174 ± 13
	133	5	5.04 ± 0.37[5]	17.4 ± 5.4[5]	915 ± 210[3]
Soybean hydrolyzate	0	4	3.55 ± 0.17	5.1 ± 0.4	180 ± 14
	115	4	4.61 ± 0.25[5]	14.9 ± 3.5[5]	709 ± 184[4]
Lactalbumin	0	8	3.95 ± 0.13	6.9 ± 0.7	271 ± 22
	123	9	5.53 ± 0.29[3]	20.1 ± 2.4[3]	1125 ± 165[3]
Lactalbumin hydrolyzate	0	4	3.81 ± 0.11	5.9 ± 0.2	212 ± 47
	120	4	5.06 ± 0.42[5]	20.1 ± 4.9[5]	1076 ± 323[5]

[1] Casein (16% "Vitamin Free" Casein, Nutritional Biochemicals Corp., Cleveland, Ohio).
Casein Hydrolyzate (15.8% "Vitamin Free" Casein Hydrolyzate (Acid), Nutritional Biochemicals Corp., + 0.2% DL-tryptophan).
Soybean (16% Promine, Central Soya Co., Inc., Chicago, Illinois).
Soybean Hydrolyzate (16% Soy Hydrolyzate (Enzymatic), Nutritional Biochemicals Corp.).
Lactalbumin (16% Lactalbumin, Nutritional Biochemicals Corp.).
Lactalbumin Hydrolyzate (16% Lactalbumin Hydrolyzate (Enzymatic), Nutritional Biochemicals Corp.).
[2] Mean value ± SE of mean.
[3] $P < 0.01$ (highly significant).
[4] $P > 0.05$ (not significant).
[5] P between 0.01 and 0.05 (probably significant).

TABLE 8

Liver protein and incorporation of radioactive amino acids into liver protein of rats fed casein diet with or without orotic acid for 3 days

Radioactive amino acid	Orotic acid	No. of rats	Liver		
			Protein	Specific activity[1]	Total radioactivity
			mg/100 g body wt	*count/min/ mg protein*	*count/min × 10⁻³/ 100 g body wt*
L-Leucine	—	3	936 ± 31[2]	21.1 ± 0.8[2]	19.8 ± 1.4[2]
	+	2	1021 ± 52	16.9 ± 0.9	17.2 ± 0
DL-Phenylalanine	—	3	853 ± 50	22.1 ± 2.1	18.8 ± 1.7
	+	3	1005 ± 52	22.2 ± 0.4	22.2 ± 0.1

[1] Specific activity (radioactivity per mg protein).
[2] Mean ± SE of mean.

probably not responsible for the differences in response of the rat liver to orotic acid when the pyrimidine is given either with casein or with casein hydrolyzate.

To determine whether the results with orotic acid with casein hydrolyzate are due to some delay in the response of the animal rather than to a qualitative change, rats were fed ad libitum casein hydrolyzate or casein diets with or without orotic acid for 7 days instead of 3 days. Also, in the same experiment, other types of proteins were used in place of casein to determine the specificity of casein in facilitating the induction of the orotic acid fatty liver. Rats fed the casein-orotic acid diet had a marked increase in liver lipid while rats fed the casein hydrolyzate-orotic acid diet had only a small increase in liver lipid (table 7). Rats fed soybean or lactalbumin and also their hydrolyzates plus orotic acid developed marked increases in liver lipid. These results indicate that orotic acid with other proteins, as well as with casein in the diet, is capable of inducing a marked fatty liver. However, although orotic acid with other protein hydrolyzates induces a severe fatty liver, similar to that with the intact protein, orotic acid with casein hydrolyzate induces only a small increase in liver lipid.

The protein content of the livers of the animals force-fed the basal casein or the orotic acid casein diet was determined in several experiments (table 8). The liver protein content of animals fed the experimental diet was slightly greater than that in the animals fed the control diet. Protein synthesis in the liver, as measured by radioactive amino acid incorporation into liver protein, was also studied (table 8). The results, expressed as specific activity or as radioactivity per total liver protein per 100 g body weight, indicate little or no difference between the control and experimental animals.

TABLE 9

Summary of major observations

Orotic acid administered to or with:	Induction of fatty liver
Starved	–
Fed rats	+
Rats receiving orotic acid and diet separately	–
10.6 g of diet	+
5.3 g of diet	–
Casein diet	+
Casein hydrolyzate or amino acid diet	–

DISCUSSION

The major observations of this study are summarized in table 9. The results indicate that orotic acid per se does not induce a fatty liver but must be administered in the diet to do so. Furthermore, the nature and quantity of the diet profoundly alters the response of the rat to the feeding of orotic acid. These results suggest that the fatty liver occurring in animals fed orotic acid is not due to a direct hepatotoxic action of the pyrimidine but rather to some more subtle metabolic imbalance induced by this compound.

The nature of the metabolic derangement is still obscure. The fatty liver induced by orotic acid resembles to some degree that induced by the administration of ethionine (11). In both, the lipid accumulating is largely triglyceride (neutral lipids) (12, 13) with a periportal lobular distribution (1, 2, 11) and the concentration of plasma lipids is markedly decreased (12, 14–17). The immediate cause for the accumulation of triglyceride in the liver with ethionine and several other agents appears to be a block in the transfer of hepatic triglyceride to the plasma (15). The same basic disturbance appears to be responsible for the accumulation of triglyceride in the liver of the rat fed orotic acid (17).

Assuming this as a working hypothesis, the question arises as to the nature of the underlying biochemical derangement responsible for this block in the lipid transfer. In the case of ethionine, Shull (18) has reported a rapid and marked decrease in the level of liver adenosine triphosphate (ATP) following acute ethionine administration. Earlier studies (19, 20) have demonstrated that acute ethionine administration inhibits liver protein synthesis. Farber et al. (21) have speculated that the pathogenetic mechanism for the block in lipid transfer in ethionine induced fatty liver may be due to the inhibition of hepatic protein synthesis which is a consequence of hepatic ATP deficiency. This concept is supported by observations that the admin-

istration of ATP or of adenine counteracts the decrease in hepatic ATP concentration (18), the inhibition of hepatic protein synthesis (22), as well as the induction of fatty liver by ethionine (21).

The results of experiments with orotic acid indicate that the mechanism by which orotic acid acts is basically quite different than that of ethionine. First, Shull[12] measured liver ATP levels in some of our experimental animals that were fed the basal casein diet containing orotic acid for 3 to 21 days and no change was noted in the content of the liver ATP in comparison with livers of animals fed the control diet. This is of special interest in that Handschumacher et al. (2) reported that the addition of adenine to the orotic acid-casein diet prevents the fatty liver. Second, this study indicates that rats fed orotic acid in the diet showed no inhibition of hepatic protein synthesis. Hankin (23) has reported that liver protein actually increases as the fatty liver develops. These indicate clearly that a different mechanism probably is involved in accounting for the block in lipid transfer from the liver in rats treated with ethionine than that in rats fed orotic acid.

The results of this study stress the importance of nutritional or metabolic imbalance in the pathogenesis of orotic acid fatty liver. This is supported by some similarities of our present results and those observed with amino acid deficiencies. In earlier acute essential amino acid deficiency studies (6, 24–26) we found that the quantity and the composition of the purified diet were important in the induction of a periportal fatty liver. In one of these studies (26) we observed that rats force-fed for 3 days a threonine-free diet developed fatty livers, but did not do so when fed a protein-free diet for the same period of time. The pathologic changes observed with certain essential amino acid deficiencies were therefore interpreted as arising due to a nutritional imbalance created by a number of dietary factors. These observations are similar to those in our present study where the quantity and composition (including protein content) of the purified diet were found to be of great importance in the induction of orotic acid fatty liver. It is conceivable that orotic acid fed in elevated quantities creates an imbalance only when other dietary components are present in the diet and that this imbalance is, in some as yet unexplained way, responsible for the fatty liver.

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Fatty Acid Composition of Plasma and Liver Lipid Components as Influenced by Diet in the Growing Chick[1]

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ABSTRACT The influence of dietary protein and cholesterol on plasma and liver lipids and on their component fatty acid composition was studied in growing chicks. Increased dietary protein decreased plasma and α-lipoprotein lipid levels and increased liver cholesterol. Cholesterol supplementation elevated plasma and liver lipids but decreased α-lipoprotein lipids. In general, feeding a low-protein or a cholesterol-supplemented diet resulted in similar fatty acid changes. Both treatments resulted in significantly increased oleic and decreased arachidonic acid levels in the plasma cholesterol esters, glycerides and phospholipids. The oleic acid level of the liver lipids was also higher in chicks fed the low-protein or cholesterol-supplemented diets. The dietary treatments tended to decrease linoleic acid levels particularly in the cholesterol ester fraction. The cholesterol ester fraction showed the most consistent changes.

The hypocholesterolemic effect of unsaturated dietary fat and fatty acids (1) has stimulated interest in the study of fatty acid metabolism. The advent of suitable procedures for routine fatty acid analyses (2, 3) has enabled more precise studies of the fatty acid composition of tissue lipids and the influence of dietary factors thereon.

Luddy et al. (3) showed that the cholesterol esters, glycerides and phospholipids of human atheroma contained greater amounts of oleic acid and less di- and tetraenoic acids than did comparable fractions from normal human sera. More recently, Schrade et al. (4) have observed higher levels of palmitoleic and oleic acids accompanied by decreased linoleic and arachidonic acid levels in the serum lipids of hyperlipemic individuals as compared with normals. Böttcher and Woodford (5) observed little difference in the fatty acid composition of serum lipids between atherosclerotic patients and normal subjects; a decreased linoleic acid level could, however, be established for the atherosclerotic subjects.

In animal studies, Fisher et al. (6) and Fisher and Feigenbaum (7) demonstrated decreased di- and tetraenoic acid levels in lipids from plaques of chicken aortas as compared with the clear areas of these tissues. Blomstrand and Christensen (8) extended these observations by showing that aortic cholesterol esters of cholesterol-fed cockerels contain relatively more oleic acid than do esters from aortas of control animals not fed cholesterol. Leveille et al. (9) have presented data indicating that in the hypercholesterolemic chick the level of oleic acid is increased, whereas the linoleic and arachidonic acid levels are decreased in the plasma and liver lipids. The most marked changes were observed in the cholesterol esters with lesser changes in the glycerides and phospholipids.

The data reported herein were obtained in an effort to determine the influence of endogenous (produced by a low-protein diet) and exogenous (resulting from cholesterol feeding) hypercholesterolemia on the fatty acid composition of plasma and liver cholesterol esters, glycerides and phospholipids.

EXPERIMENTAL

Male Hy-line White Leghorn chicks were fed a commercial diet for one week and were then distributed to the various experimental groups on the basis of body weight. Eight chicks were distributed in such a manner as to have almost identical mean weights for all groups. The chicks were

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[1] The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

housed in heated cages having raised wire floors. Food and water were supplied ad libitum. Body weight and food consumption were determined weekly.

The composition of the basal diet was as follows: (in g/100 g of diet) Sesame oil meal (46.8% protein, N × 6.25), 25.64; corn oil, 5.00; salt mix,[2] 4.00; vitamin mix[2], 0.40; choline chloride, 0.20; L-lysine·HCl (99% purity), 0.87; glucose to 100. Additional sesame oil meal was added to the diet of the high-protein groups to supply 25% protein. Cholesterol, when fed, was added at a level of 0.3 and 2.0% to the low-protein and high-protein diet, respectively. These levels of cholesterol used were selected to produce a similar degree of hypercholesterolemia at each of the protein levels used (6). All additions to the basal diet were made at the expense of glucose.

At the end of the 4-week experimental period, blood was drawn by cardiac puncture using a heparinized syringe. The animals were killed and the liver excised. The livers and plasma were frozen and stored at −20°C until analyzed.

Plasma cholesterol, lipid phosphorus and glycerides were determined as described previously (10). β-Lipoproteins were precipitated (11) and cholesterol and lipid phosphorus content of the supernatant containing the α-lipoprotein was determined as for plasma (10). Liver lipids were extracted with chloroform: methanol (2:1, v/v); after determining total liver fat gravimetrically, the lipid was dissolved in chloroform and appropriate aliquots were taken for cholesterol and lipid phosphorus analyses using the same methods as those for plasma (10).

Plasma and liver lipid extracts were kept under an atmosphere of O_2-free nitrogen to prevent oxidation. Appropriate aliquots were subjected to silicic acid chromatography for separation of cholesterol esters, glycerides and phospholipids (12). The various fractions were saponified with 0.5 N alcoholic KOH for 40 minutes after evaporation of the solvents under a stream of O_2-free nitrogen. After acidification with 1 N HCl, the saponified samples were extracted twice with ethyl ether. The combined ether extracts were washed with water and dried over anhydrous Na_2SO_4. The fatty acids were then esterified with methanol-boron trifluoride according to the procedure of Metcalfe and Schmitz (13). The methyl esters were purified by sublimation (14) and were then separated by gas chromatography using a 0.3-cm OD, 244-cm column packed with 20% DEGS (specially prepared diethylene glycol succinate (15) coated on 100 to 140 mesh Gas Chrom P.[3] The columns were operated at 200°C with a helium flow so regulated that the elution time of methyl palmitate was 11 mintues. A hydrogen flame detector was used. Peaks were identified by comparing relative retention times to known standards[4] which were determined daily. Peak areas were quantified by triangulation. Results are presented for 6 major fatty acid components only; others were observed, but generally in small and variable amounts. In most cases the 6 acids considered accounted for at least 95% of the total. The values presented in the results section are expressed as a percentage of the sum of the 6 fatty acids considered. The results obtained were statistically evaluated by means of the analysis of variance.

RESULTS

The results obtained are presented in tables 1 and 2; in tables 1 and 3 are shown the probabilities of the differences observed being statistically significant.

Dietary protein significantly improved weight gain, decreased plasma and α-lipoprotein lipids, and increased liver cholesterol (table 1). Cholesterol supplementation increased plasma lipids and liver fat and cholesterol, but depressed α-lipoprotein cholesterol and lipid phosphorus levels.

The determined fatty acid values are presented in table 2 and the probability values for the significance of observed differences are shown in table 3. In general, the differences between chicks fed a low- or high-protein level and between chicks fed a cholesterol-supplemented or cholesterol free diet are similar. Feeding a low-protein or a cholesterol-supplemented diet resulted in decreased arachidonic acid

[2] For composition, see Leveille et al. (10).
[3] Applied Science Laboratory, State College, Pennsylvania.
[4] Obtained from The Hormel Institute, Austin, Minnesota.

TABLE 1

Body weight gain and plasma, plasma α-lipoprotein and liver lipids of chicks fed different levels of protein and cholesterol; significance of interaction protein and cholesterol components of variation

	Treatment group				Source of variation[1]		
	12% Protein	12% Protein + 0.3% cholesterol	25% Protein	25% Protein + 2% cholesterol	Interaction	Protein	Cholesterol
Body wt gain, g	178 ± 36[2]	171 ± 42	320 ± 14	316 ± 34	ns	$P < 0.01$	ns
Plasma							
Cholesterol, mg/100 ml	266 ± 44	982 ± 118	251 ± 25	828 ± 170	ns	$P < 0.05$	$P < 0.01$
Lipid P, mg/100 ml	17.6 ± 3.1	20.7 ± 2.5	15.2 ± 1.4	16.6 ± 3.6	ns	$P < 0.01$	$P < 0.05$
Glycerides, mmoles/l	0.62 ± 0.18	1.48 ± 0.43	0.35 ± 0.14	0.97 ± 0.28	ns	$P < 0.01$	$P < 0.01$
Plasma α-lipoprotein							
Cholesterol, mg/100 ml	146 ± 21	128 ± 17	119 ± 28	80 ± 9	ns	$P < 0.01$	$P < 0.01$
Lipid P, mg/100 ml	13.1 ± 2.3	6.4 ± 0.9	10.0 ± 2.2	4.2 ± 0.7	ns	$P < 0.01$	$P < 0.01$
Liver							
Fat, %	6.3 ± 0.6	8.8 ± 0.5	5.1 ± 0.3	9.5 ± 1.8	$P < 0.05$	ns	$P < 0.01$
Cholesterol, mg/g	4.8 ± 0.4	31.2 ± 3.3	5.2 ± 0.5	37.4 ± 7.9	ns	$P < 0.05$	$P < 0.01$
Lipid P, mg/g	1.1 ± 0.3	1.0 ± 0.2	1.0 ± 0.2	1.1 ± 0.3	ns	ns	ns

[1] Based on analysis of variance.
[2] Mean for 8 chicks ± SD.

(C_{20}:4) levels in all plasma fractions. Chicks fed the low-protein diet also had lower arachidonic acid levels in the liver glycerides and phospholipids.

Both low-protein and cholesterol feeding also significantly increased the oleic acid (C_{18}:1) content of all plasma and liver lipid fractions with the exception of the plasma glycerides. The level of oleic acid was higher in the plasma glycerides of chicks fed either a low-protein or cholesterol-supplemented diet (table 2); however, the differences were not statistically significant (table 3). The changes in the linoleic (C_{18}:2) acid level resulting from the 2 dietary treatments were not consistent. The low-protein diet decreased the linoleic acid level in the plasma and liver cholestrol esters and the liver glycerides; in both the plasma and liver phospholipids the level of linoleic acid was higher in chicks fed the low-protein diet. Cholesterol supplementation decreased plasma cholesterol ester linoleic acid and increased the level of this acid in plasma phospholipids and liver cholesterol esters and glycerides. Cholesterol feeding also decreased the palmitic (C_{16}:0) acid content of all 3 liver lipid fractions.

DISCUSSION

The effects of cholesterol supplementation and dietary protein level on plasma and liver lipids are in accord with published reports (10, 16). In general, the changes induced in fatty acid composition by feeding either a low-protein or a cholesterol-supplemented diet are consistent and in the same direction. This becomes of significance when one considers that both of these dietaries have been shown to be hypercholesterolemic and atherogenic in the growing chick (16, 17).

The most consistent fatty acid changes observed were the increased oleic acid and decreased arachidonic acid levels noted in chicks fed a low-protein diet or cholesterol, particularly the former. The changes in linoleic acid were more variable. The most marked changes were observed in the cholesterol ester fraction. This is of particular interest when considered in light of the apparent relationship between plasma cholesterol ester

TABLE 2

Fatty acid composition of plasma and liver lipids in chicks fed different levels of protein and cholesterol

Fatty acid[2]	Dietary variables[1]			
	12% Protein		25% Protein	
	No cholesterol	0.3% cholesterol	No cholesterol	2.0% cholesterol
	% total fatty acids		*% total fatty acids*	
Plasma				
Cholesterol esters				
16:0	20.6 ± 2.6[3]	11.3 ± 3.8	15.5 ± 2.9	12.4 ± 2.8
16:1	8.6 ± 2.2	5.1 ± 1.4	5.4 ± 1.2	7.4 ± 2.0
18:0	10.2 ± 1.4	7.5 ± 1.8	10.8 ± 1.6	8.6 ± 1.5
18:1	28.0 ± 2.2	48.3 ± 4.7	16.9 ± 4.2	33.6 ± 2.9
18:2	28.4 ± 5.6	25.9 ± 4.9	38.2 ± 4.8	31.4 ± 2.9
20:4	4.2 ± 1.3	1.9 ± 1.1	13.2 ± 3.5	6.7 ± 1.9
Glycerides				
16:0	21.8 ± 3.0	21.4 ± 3.2	21.6 ± 3.2	18.5 ± 1.8
16:1	8.3 ± 2.9	7.2 ± 2.1	6.8 ± 3.4	3.3 ± 0.8
18:0	11.2 ± 3.1	8.8 ± 2.3	12.5 ± 3.8	9.2 ± 1.0
18:1	28.7 ± 1.7	34.6 ± 4.5	25.1 ± 1.5	26.9 ± 1.2
18:2	26.6 ± 7.8	25.6 ± 4.3	29.4 ± 6.2	37.9 ± 2.8
20:4	3.6 ± 1.3	1.6 ± 1.0	5.0 ± 1.5	4.2 ± 1.7
Phospholipids				
16:0	20.1 ± 1.2	19.5 ± 1.3	17.9 ± 1.1	17.6 ± 1.4
16:1	2.8 ± 0.7	3.5 ± 0.9	3.6 ± 1.0	3.6 ± 0.9
18:0	23.4 ± 1.1	25.2 ± 1.4	28.1 ± 1.8	26.5 ± 1.0
18:1	14.6 ± 1.2	16.1 ± 1.5	9.3 ± 0.9	10.5 ± 0.8
18:2	20.3 ± 2.8	24.8 ± 1.8	14.5 ± 1.1	20.9 ± 2.0
20:4	18.9 ± 3.6	11.1 ± 2.3	26.6 ± 2.5	21.0 ± 2.5
Liver				
Cholesterol esters				
16:0	26.4 ± 5.1	9.4 ± 3.2	28.6 ± 1.7	13.1 ± 7.3
16:1	14.5 ± 5.5	8.6 ± 2.1	22.8 ± 3.7	9.5 ± 5.3
18:0	16.2 ± 3.4	5.5 ± 1.1	13.1 ± 1.9	6.7 ± 3.8
18:1	29.4 ± 6.1	60.4 ± 6.2	23.3 ± 2.5	45.6 ± 13.2
18:2	12.3 ± 4.7	15.6 ± 3.6	13.6 ± 1.4	22.2 ± 6.5
20:4	1.1 ± 2.9	0.6 ± 1.1	tr[4]	2.9 ± 2.4
Glycerides				
16:0	27.3 ± 4.0	23.8 ± 2.8	28.5 ± 2.9	24.7 ± 1.8
16:1	8.7 ± 1.0	8.2 ± 0.8	5.1 ± 1.1	3.1 ± 3.1
18:0	12.1 ± 1.9	11.1 ± 2.3	15.6 ± 2.7	13.1 ± 3.2
18:1	37.8 ± 2.6	42.3 ± 2.1	20.0 ± 2.1	25.2 ± 1.7
18:2	12.0 ± 2.3	13.3 ± 2.5	21.9 ± 3.8	26.4 ± 3.2
20:4	2.0 ± 1.2	1.2 ± 0.9	8.8 ± 1.7	7.5 ± 2.6
Phospholipids				
16:0	15.4 ± 1.4	13.5 ± 1.1	15.6 ± 0.9	14.8 ± 2.0
16:1	2.6 ± 3.4	3.7 ± 1.0	0.7 ± 1.3	1.2 ± 1.3
18:0	27.3 ± 3.4	26.6 ± 1.9	28.4 ± 2.3	25.3 ± 3.0
18:1	14.1 ± 3.9	16.2 ± 2.1	7.7 ± 1.3	10.7 ± 1.5
18:2	21.9 ± 5.0	24.2 ± 2.3	19.0 ± 1.1	21.0 ± 2.5
20:4	18.6 ± 4.9	15.9 ± 3.0	28.6 ± 3.6	27.0 ± 3.5

[1] Values are reported as a percentage of the 6 acids shown.
[2] First number refers to carbon chain length; number after colon refers to number of double bonds.
[3] Mean of 8 determinations ± SD.
[4] Trace, amount insufficient for accurate determination.

TABLE 3

Significance of interaction, protein and cholesterol levels as sources of variation in fatty acid composition of plasma and liver lipids[1]

Fatty acid[2]	Source of variation		
	Interaction	Protein level	Cholesterol
Plasma			
Cholesterol esters			
16:0	$P < 0.01$	ns	ns
16:1	$P < 0.01$	ns	ns
18:0	ns	ns	$P < 0.01$
18:1	ns	$P < 0.01$	$P < 0.01$
18:2	ns	$P < 0.01$	$P < 0.05$
20:4	$P < 0.05$	$P < 0.01$	$P < 0.01$
Glycerides			
16:0	ns	ns	ns
16:1	ns	$P < 0.01$	$P < 0.05$
18:0	ns	ns	$P < 0.01$
18:1	$P < 0.05$	ns	ns
18:2	$P < 0.05$	ns	ns
20:4	ns	$P < 0.01$	$P < 0.01$
Phospholipids			
16:0	ns	$P < 0.01$	ns
16:1	ns	ns	ns
18:0	$P < 0.01$	ns	ns
18:1	ns	$P < 0.01$	$P < 0.01$
18:2	ns	$P < 0.01$	$P < 0.01$
20:4	ns	$P < 0.01$	$P < 0.01$
Liver			
Cholesterol esters			
16:0	ns	ns	$P < 0.01$
16:1	$P < 0.05$	ns	ns
18:0	$P < 0.05$	ns	ns
18:1	ns	$P < 0.01$	$P < 0.01$
18:2	ns	$P < 0.05$	$P < 0.01$
20:4	$P < 0.05$	ns	ns
Glycerides			
16:0	ns	ns	$P < 0.01$
16:1	ns	$P < 0.01$	ns
18:0	ns	$P < 0.01$	ns
18:1	ns	$P < 0.01$	$P < 0.01$
18:2	ns	$P < 0.01$	$P < 0.05$
20:4	ns	$P < 0.01$	ns
Phospholipids			
16:0	ns	ns	$P < 0.05$
16:1	ns	$P < 0.01$	ns
18:0	ns	ns	ns
18:1	ns	$P < 0.01$	$P < 0.01$
18:2	ns	$P < 0.01$	ns
20:4	ns	$P < 0.01$	ns

[1] Based on analysis of variance.
[2] First number refers to carbon chain length; number after colon refers to number of double bonds.

arachidonic acid level and susceptibility to atherosclerosis (18).

The dietary fat used in the present diets (corn oil) had a high linoleic acid content; it should therefore be kept in mind that differences in food intake and, consequently, fat intake may have influenced the results obtained. However, the changes noted cannot be totally attributed to differences in fat intake, since the food con-

sumption at each protein level was not influenced by cholesterol supplementation.

Previous reports have shown plasma or serum and tissue alterations of oleic-linoleic and arachidonic acid levels in animals and humans known to have atherosclerosis or some derangement of lipid metabolism (19–21). The data herein reported are generally in accord with these reports. The reports cited and the present data could be interpreted to indicate that altered oleic-linoleic-arachidonic acid levels are an indication of abnormal lipid metabolism.

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Amino Acid Balance and Imbalance

XII. EFFECT OF AMINO ACID IMBALANCE ON SELF-SELECTION OF DIET BY THE RAT[1]

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ABSTRACT Observations of some effects of an amino acid imbalance on food intake and food selection of rats in different physiological states are described. The imbalance was created by adding a mixture of amino acids lacking histidine to a purified diet containing 6% of beef fibrin. Food intake was depressed when the diet in which there was an imbalance of amino acids was substituted for one containing balanced protein; and rats given a choice between the "imbalanced" diet and a protein-free diet showed a decided preference for the latter over the former. This preference became evident more quickly with nondepleted and with starved rats than had previously been observed with protein-depleted rats. Neither protein-depleted nor non-depleted rats showed a specific preference for either of 2 balanced diets which differed in nutritive value, but both groups showed a clear preference for a more balanced diet over an imbalanced diet when the 2 diets differed by only 0.05 to 0.1% of L-histidine·HCl.

Dietary amino acid pattern can affect food selection (1) and food intake (2, 3). Protein-depleted rats given a choice between a protein-free diet and a diet in which there is an imbalance of amino acids select the latter for 2 to 3 days, presumably until they are repleted; then they reject it and select mainly the protein-free diet (1). Although the initial preference for the diet in which there is an imbalance of amino acids can be related to its adequacy for repletion compared with the protein-free diet, the subsequent preference for a diet which cannot support growth must have some other basis.

The change in diet preference and the depression in food intake of rats ingesting a diet in which there is an imbalance of amino acids are associated with changes in the plasma amino acid pattern of the rats (2, 3). As a working hypothesis, it has been suggested that some homeostatic mechanism which is sensitive to the altered plasma or tissue amino acid pattern may depress food intake and lead to a preference for the protein-free diet which produces a more balanced plasma and tissue amino acid pattern despite its inability to prevent loss in weight. The fact that plasma amino acid pattern is not so severely altered by a dietary imbalance of amino acids during the repletion period when the imbalanced diet is preferred would be in accord with this hypothesis.

Experiments reported in this paper were carried out to determine how other physiological conditions that can alter plasma amino acid concentrations, such as starvation or full feeding, influenced selection of a diet in which there was an imbalance of amino acids. The ability of the rat to discriminate between 2 diets, one in which there was an imbalance of amino acids and another in which the imbalance was corrected by adding only 0.1% or less of the missing amino acid, was also examined.

EXPERIMENTAL

Male rats of the Holtzman strain, weighing 110 to 120 g, were used in all experiments. The rats were housed in

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individual suspended cages with screen bottoms. They were fed as described below; water was offered ad libitum.

In the experiments on diet selection, identical food containers from which there was little spillage were placed at random in the cages; the containers were changed twice a week during the experiments. In all experiments, food intakes were measured and recorded daily, and the animals were weighed twice a week. Groups of 6 rats each were used unless it is otherwise indicated.

Protein-depleted rats. Rats were depleted of protein by feeding them a protein-free diet for 6 days. Animals that lost 15 to 20 g, the average loss for such animals, were selected for the experiments.

Starved rats. Rats were starved by giving them only water for 3 days; those that lost 30 to 35 g, the average loss for such animals, were chosen for the experiments.

Diets. The basal diet was that described by Kumta et al. (4). This contained: fibrin, 6; salt mixture (5), 5; corn oil, 5; vitamin supplement (5), 0.25; choline chloride, 0.15 and "dextrin" (moist corn starch heated at 121° in an autoclave for 3 hours, then dried and ground) as the carbohydrate to make 100%. Fat-soluble vitamins were added to the corn oil to provide the following concentrations per 100 g of the diet: vitamin A, 400 IU; vitamin D, 40 IU; and vitamin E, 10 mg. The protein-free diet was of the same composition as the basal diet, except that dextrin replaced fibrin. Additions of amino acids were compensated for by adjusting the percentage of dextrin. All diets were stored in a refrigerator.

An amino acid imbalance was created by adding to the basal diet a mixture of amino acids lacking histidine ("imbalanced" diet). The amino acid mixture provided in per cent of the diet: DL-methionine, 0.4; DL-phenylalanine, 0.6; L-leucine, 0.6; DL-isoleucine, 0.4; DL-valine, 0.4; L-lysine·HCl, 0.6; L-arginine, 0.2; DL-threonine, 0.4; and DL-tryptophan, 0.2. The "corrected" diet was made by adding to the imbalanced diet 0.1% of L-histidine·HCl. In some experiments, as will be indicated, a corrected diet with only 0.05% of L-histidine·HCl was used; this percentage is sufficient to prevent the imbalance (6).

RESULTS

Values for food intake of nondepleted rats fed ad libitum the "imbalanced," the

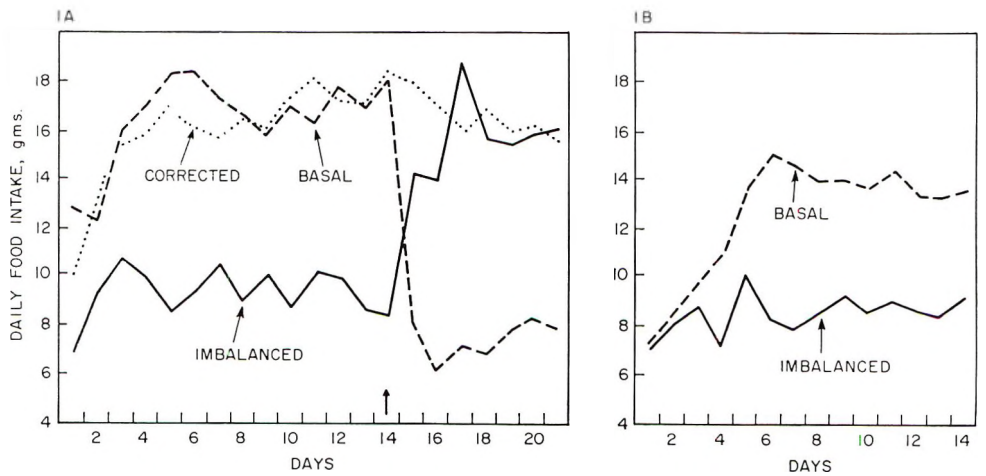


Fig. 1 Average daily food intake.

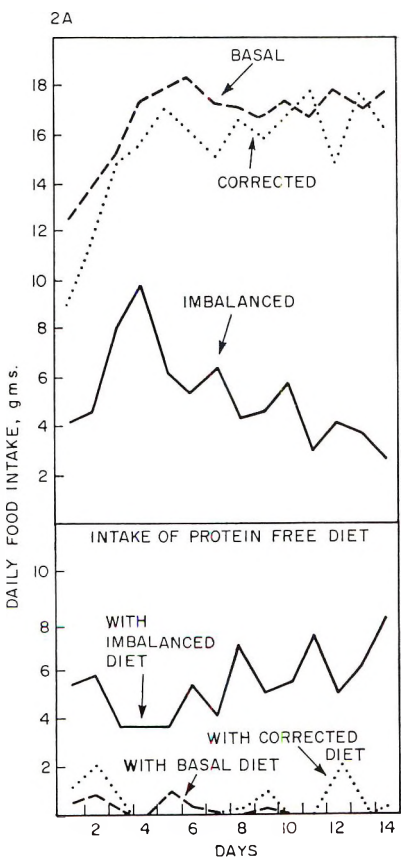
A Nondepleted rats fed basal, "imbalanced" or "corrected" diet. After 2 weeks the diets of the groups initially fed the "imbalanced" and the "corrected" diets were exchanged as indicated by the arrow.

B Rats previously starved for 3 days then fed basal or "imbalanced" diet.

basal or the "corrected" diet are plotted in figure 1A. The food intake of the "imbalanced" group was depressed on the first day; after 2 weeks the food consumption of this group was about 50% of those of the other 2 groups. At the end of 2 weeks when the group receiving the basal diet was given the "imbalanced" diet, the food intake of this group promptly decreased. When the group receiving the "imbalanced" diet was given the "corrected" diet, the food intake of this group promptly increased.

Figure 1B shows the depression in food intake produced by feeding the "imbalanced" diet to starved rats; the food consumption of the group fed the basal diet ad libitum for 2 weeks increased progressively until the sixth day and remained more or less constant thereafter. During the first 2 days the food consumption of the group fed the "imbalanced" diet, was only a little less than that of the basal group but subsequently was only about 60% of that of the basal group.

Selection between imbalanced and protein free diets. The feeding behavior of nondepleted rats offered a choice between the basal, the "imbalanced" or the "corrected" diet and a protein-free diet, both offered ad libitum for 2 weeks, is shown in figure 2A. The groups offered the basal or the "corrected" diet ate these diets almost exclusively throughout the experiment and rejected the protein-free diet. The group given a choice between the "imbalanced" and the protein-free diet, ate both diets but after the fourth day the intake of the "imbalanced" diet decreased progressively. By the seventh day, 2 rats rejected the "imbalanced" diet completely and ate only the protein-free diet, and at the end of the experiment 3 rats were eating only this diet.



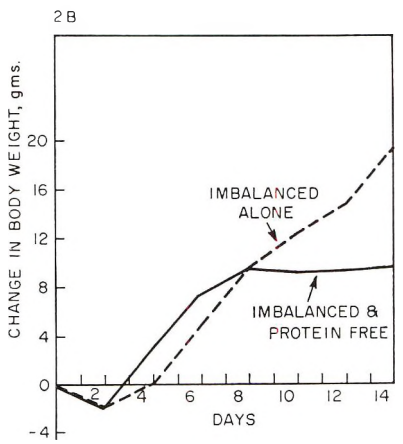


Fig. 2 Food intake and weight change of nondepleted rats fed various diets concurrently with protein-free diet.

A Intake of specific diet, above; intake of protein-free diet, below.

B Change in body weight of rats fed "imbalanced" diet with or without protein-free diet.

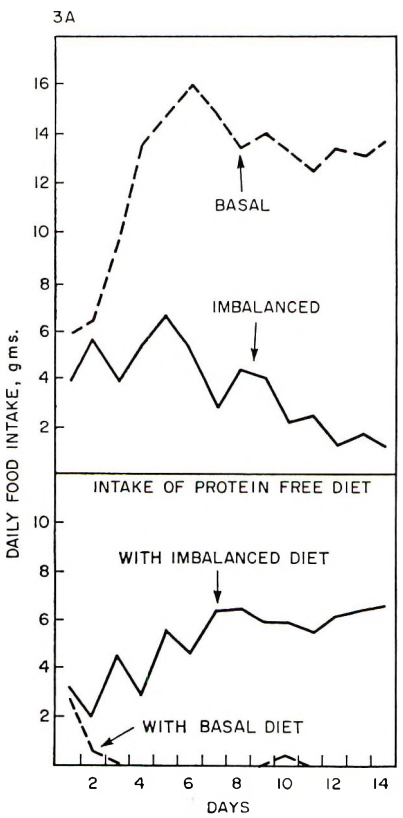
The changes in weight of nondepleted rats offered the "imbalanced" diet alone, ad libitum, or simultaneously with the protein-free diet, are shown in figure 2B. Owing to their preference for the protein-free diet, those offered a choice gained only one-half as much as those offered no choice.

The feeding behavior of starved rats was similar to that of nondepleted rats and differed from that reported previously for protein-depleted animals (1). Figure 3A shows that starved rats selected some of the protein-free diet on the first day when this diet was offered simultaneously with the balanced or the "imbalanced" diet; at the end of the 2-week period, 2 rats were rejecting the imbalanced diet completely and the others were eating only a small amount of it. The rats, offered the basal diet and the protein-free diet simultaneously, ate only the basal diet after the first day.

The effect of selection of the protein-free diet over the "imbalanced" diet on the body weight of starved rats is shown in figure 3B. Owing to their preference for the protein-free diet, the starved rats given a choice between the 2 diets gained only one-half as much as those offered no choice.

Selection between imbalanced and balanced diets. The feeding behavior of protein-depleted rats offered a choice between the "imbalanced" diet and the basal diet is shown in figure 4A. Some preference for the basal diet was evident on the first 2 days, and after the third day they ate almost exclusively the basal diet. Similar results were obtained with nondepleted rats when the same choice was given (fig. 4B), but rejection of the imbalanced diet appeared to occur more rapidly for by the second day the basal diet made up 91% of the food consumed.

In an effort to obtain information about the ability of the rat to discriminate be-



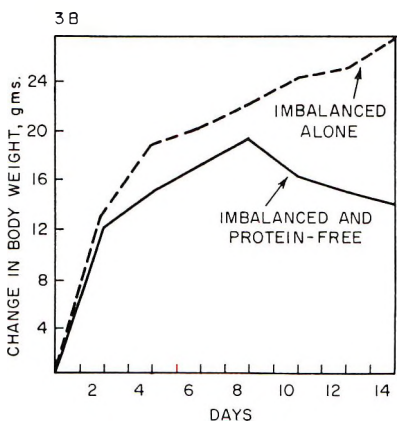


Fig. 3 Food intake and weight change of starved rats fed basal or "imbalanced" diet concurrently with protein-free diet.

A Intake of specific diet, above; intake of protein-free diet below.

B Change in body weight of rats fed "imbalanced" diet with or without protein-free diet.

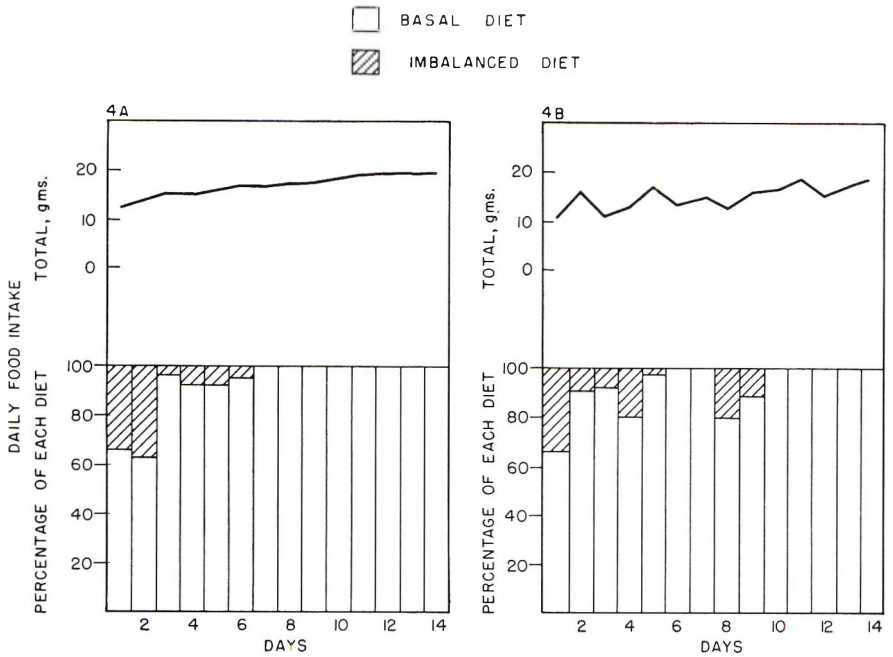


Fig. 4 Food intake of rats fed "imbalanced" and basal diets concurrently. Total food intake, above; percentage of total food from each diet, below.

A Protein-depleted rats.
B Nondepleted rats.

tween 2 balanced diets differing in nutritive value, the above experiment was repeated using the "corrected" diet in place of the "imbalanced" diet. Both diets offered were balanced diets, with the "corrected" presumably of about the same palatability as the "imbalanced" diet but having a higher nutritive value. The behavior of the rats in this experiment (table 1) was not uniform; 4 of 6 protein-depleted rats selected and ate only the "corrected" diet for the 14 days, but the other 2 selected the basal diet. The nondepleted rats behaved similarly; 3 selected the "corrected" diet and the other 3, the basal diet. After 2 weeks the "corrected" diet was replaced by the "imbalanced" diet, then the animals that had previously selected the "corrected" diet changed their preference within 2 or 3 days and began eating the basal diet.

In the experiments so far described, differences in taste and texture between the basal and amino acid supplemented

TABLE 1

Food intake of protein-depleted and nondepleted rats offered the basal and the "corrected" diets concurrently for 2 weeks

Rat no.	Food consumption			
	Protein-depleted rats		Nondepleted rats	
	Basal	Corrected	Basal	Corrected
	g/2 wk	*g/2 wk*	*g/2 wk*	*g/2 wk*
1	2.1	177.6	200.6	3.1
2	3.7	213.5	195.5	8.5
3	3.0	214.7	3.9	210.5
4	239.0	3.6	2.1	198.7
5	212.2	4.5	1.0	212.8
6	1.5	203.2	205.6	2.7

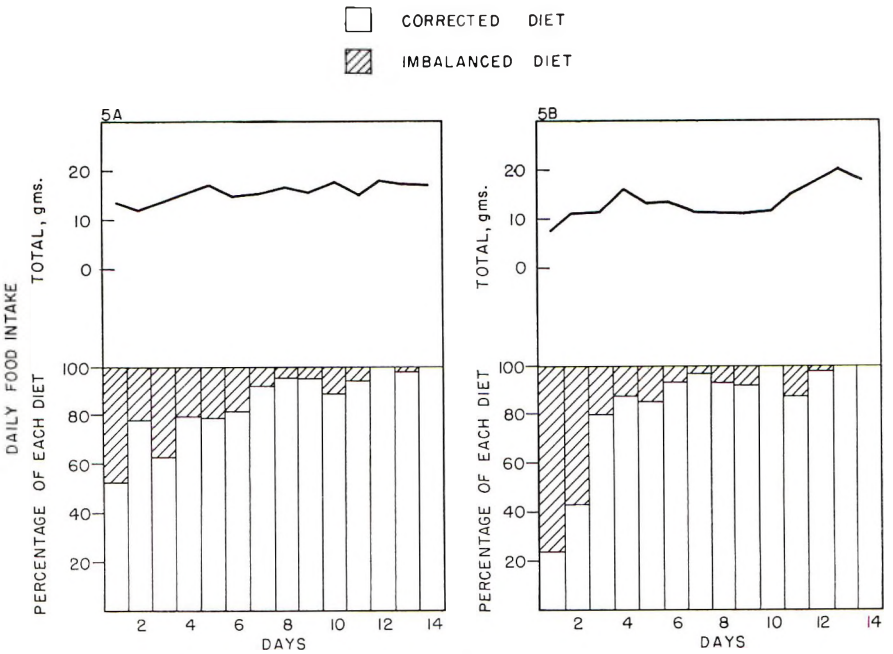


Fig. 5 Food intake of rats fed "imbalanced" and "corrected" diets concurrently. Total food intake, above; percentage of total food from each diet, below.

A Protein-depleted rats. ("Corrected" diet indicates "imbalanced" diet + 0.1% L-histidine·HCl.)

B Nondepleted rats. ("Corrected" diet indicates "imbalanced" diet + 0.05% L-histidine·HCl.)

diets offered to the rats probably made it easy for them to identify a particular diet prior to selection. In an effort to establish the capacity of the rat to select between 2 diets that were similar in taste and texture, one balanced and the other "imbalanced," the following experiments were done. A group of 10 protein-depleted rats was offered a choice between the "imbalanced" diet and the "corrected" diet containing 0.1% of histidine·HCl; both diets were offered ad libitum for 2 weeks, and the food containers were changed twice a week to prevent the rats from identifying them. As shown in figure 5A, during the first day the animals ate 53% of their total food in the form of the "corrected" diet; by the second day the percentage had increased to 78%, and thereafter the rats successfully selected mainly the "corrected" diet. By the seventh day, 5 of the 10 rats completely rejected the "imbalanced" diet without eating a measurable amount of it; by the tenth day 9 out of the 10 rats in the group did so.

The same experiment was performed with nondepleted rats, but in this case the "corrected" diet contained only 0.05% of histidine·HCl, just sufficient to prevent the imbalance (6). During the first 2 days the rats did not select the "corrected" diet as they consumed 76 and 57%, respectively, of their daily intake in the form of "imbalanced" diet; but by the third day, they began to discriminate between the 2 diets quite well (fig. 5B). By the third day 2 rats completely rejected the "imbalanced" diet; and by the seventh day, 9 of the 10 rats did so, and by the end of the experiment all were rejecting it.

DISCUSSION

Physiological state and diet selection. The results obtained with nondepleted rats differ only in the time of the effect from those obtained previously with protein-depleted rats (1, 2). The food intake of nondepleted rats fed the "imbalanced" diet ad libitum decreased on the first day to about 50% of that of the group fed the

basal diet, whereas only after the third day did the food intake of protein-depleted rats on the same regimen decrease sharply. Also, nondepleted rats offered a choice between the "imbalanced" diet and the protein-free diet ate a considerable amount of the protein-free diet on the first day, whereas protein-depleted rats offered a similar choice ate very little of the protein-free diet until after the third day.

Differences in palatability among the diets would not appear to account for these effects because, although the protein-free diet was selected over the "imbalanced" diet, the "corrected" diet (which closely resembles the "imbalanced" diet in composition and texture and presumably in palatability) was selected over both the protein-free and the "imbalanced" diets. Also, the preference for the protein-free diet over the "imbalanced" diet cannot be attributed to any superiority in the nutritive value of the protein-free diet because it does not support growth, whereas the "imbalanced" diet does.

The altered feeding behavior of protein-depleted and nondepleted rats is associated with a change in the plasma free amino acid pattern of the animals (2). Concentrations of all indispensable amino acids in plasma except that of histidine (which decreases) increase more rapidly in nondepleted rats than in protein-depleted rats, and the ratios of these to histidine increase even more rapidly. Homeostatic mechanisms that tend to prevent the pattern of plasma amino acids from becoming still more unbalanced may be activated when these ratios reach certain critical values. This would apparently occur on the first day in nondepleted rats, but only on the third day in protein-depleted rats. Selection of a protein-free diet over an "imbalanced" diet at this time could be related to the ability of the protein-free diet to produce a more balanced plasma amino acid pattern even though it does not support growth. The observation that rats consume more of a protein-free diet than of a diet completely lacking one amino acid (7) could also be a result of such a mechanism, as could the observation that food intake is depressed when the "imbalanced" diet is offered alone.

The feeding behavior of starved rats offered the "imbalanced" diet concurrently with a protein-free diet resembles more closely the behavior of nondepleted than of protein-depleted rats. This suggests that the critical point at which food intake is depressed is reached more rapidly in starved and nondepleted than in protein-depleted rats and may account for the observation of Kumta and Harper (8) that protein-depleted rats fasted for 12 hours or more before being fed the "imbalanced" diet show a depression in food intake much earlier than do protein-depleted rats not starved for this period (1).

Selection between imbalanced and balanced diets. Failure of rats to discriminate between the "corrected" and the basal diets despite the better nutritive value of the "corrected" diet suggests that discrimination against the "imbalanced" diet when it was offered with the basal diet was not a consequence of the lower nutritive value of the "imbalanced" diet but of some specific physiological effects it produced. The basal and the "corrected" diets both produce balanced plasma amino acid patterns, which may explain why there is no specific selection of one of these diets over the other. Replacement of the "corrected" by the "imbalanced" diet results in altered blood amino acid pattern and preference for the basal diet.

The last experiments described show that both protein-depleted and nondepleted rats have an amazing ability to discriminate between 2 diets differing only by 0.1 or 0.05% of histidine·HCl. It was apparently more difficult for them to discriminate when the difference in histidine content was smaller. As the lower amount will correct the imbalance, the difficulty is apparently in the ability of the rat to identify the "corrected" diet with the lower histidine content. Franke and Potter (9) reported that rats discriminated against diets that were toxic due to a selenium content of only 30 ppm; and Monty and Click (10) reported that rats given a choice between 2 diets differing only by 800 ppm of molybdenum rejected the one containing the toxic amount of molybdenum after a short period even though the diets were very similar in palatability.

These observations indicating that the rat has a remarkable ability to select the more beneficial of 2 diets are not in accord with the observations of some workers (11–13) who used different diets, nor are they in accord with the observation that the rat will select a protein-free diet in preference to an imbalanced diet (1). The lack of accord is not unexpected if the following relationships are considered: 1) When adverse effects are not produced rapidly by any of the diets offered, self-selection appears to be regulated only by unexplained individual preferences of the rat and to be unrelated to differences in nutritive value among the diets. Such behavior was observed when the basal and corrected diets were offered simultaneously (table 1) and resembles that cited above (11–13). 2) When the animal is depleted of some nutrient, selection of a diet that contains the missing nutrient might be expected if ingestion of the diet quickly results in a feeling of well-being. This appears to be the case in experiments in which the imbalanced diet was selected instead of the protein-free diet by the protein-depleted rat, and is in accord with results reported by Scott and Quint (14), Young (15) and Harris et al. (16). 3) When some adverse or toxic effect is rapidly produced by any of the diets offered, rejection of the diet might be expected. This type of behavior was observed in nondepleted animals, in which the imbalanced diet, which was not toxic per se, but which produced marked changes in the blood amino acid pattern, was rejected. The observations on the rejection of diets containing a toxic level of selenium or molybdenum also fit this pattern.

These conclusions support the hypothesis that nutritional choice is brought about more by learning, according to Harris et al. (16) and Yudkin (17), than by some inner drive or instinct, as was suggested by Richter (18). For the rat to identify the imbalanced diet and, after a learning period, reject it without consuming a readily measurable amount, it must be assumed that during the learning period some sensory response to the diet can be associated with a physiologic disturbance, possibly owing to or reflected in the altered pattern of blood amino acids. Once the association is definitively established, the rat can avoid the imbalanced diet, or select the corrected diet independently of its physiologic effect. This would be in accord with the postulation of Le Magnen (19) that the first step in developing a special appetite for, or refusal of, a diet would be sensory recognition; the second, association of this characteristic with the physiological effects of the diet; and finally, the acceptance or the rejection of all diets having that sensory characteristic.

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Vitamin B_{12} and DNA Biogenesis[1]

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ABSTRACT The enzyme, methylene tetrahydrofolic dehydrogenase, which mediates the reduction of one-carbon fragments from the formate to the formaldehyde level in the metabolic pathway leading to thymine methyl biogenesis, is reduced in activity in vitamin B_{12}-deficient chick livers. The addition of vitamin or coenzyme B_{12} in vitro under aerobic conditions stimulates the deficient enzyme preparations with a K_m of 2.9×10^{-10} M. A test of the physiological significance of this stimulation has been made. Control and vitamin B_{12}-deficient chickens were injected with tracer amounts of formate-C^{14} and formaldehyde-C^{14}. With either tracer the specific activity of the purines and of thymine was diminished in the bone marrow of the deficient chicks, but the percentage decrease in the specific activity of thymine was greater than the percentage decrease in the purines. No difference was observed between the 2 tracers. It is concluded that a form of vitamin B_{12} plays a significant role in DNA synthesis, probably in the biogenesis of the thymine methyl.

Previous reports from this laboratory have shown that vitamin B_{12} stimulates the conversion of formate into thymine methyl in *Lactobacillus leichmannii* and in chick bone marrow suspensions (1–3). On the other hand the uptake of labeled formaldehyde, glycine, serine and methionine methyl into thymine methyl is not affected by vitamin B_{12} in these systems. Noronha and Screenivasan (4) report that the addition of vitamin B_{12} to rat diets suppresses the conversion of formate into the purines of liver acid-soluble nucleotides, and the experiments of Arnstein (5,6) demonstrate that vitamin B_{12} administered to rats increases the conversion of formate to choline methyl groups, serine and methionine and decreases the formate uptake into liver RNA purines. It has been suggested as a result of these observations that one of the functions of vitamin B_{12} may be in the reduction of one-carbon compounds between the formate and formaldehyde levels of oxidation. This reaction is mediated by the enzyme hydroxymethyl tetrahydrofolic dehydrogenase (7), more properly called methylene tetrahydrofolic dehydrogenase (8).

The experiments to be described in this report were designed to determine whether vitamin B_{12} deficiency has any effect on the level of activity of methylene tetrahydrofolic dehydrogenase in chick livers, and if so, if the addition of vitamin B_{12} in vitro increases the activity in the deficient tissue. Finally, a check of the physiological significance of the relation of vitamin B_{12} to this enzyme has been made. A brief report of part of these observations has been made.[3,4,5]

EXPERIMENTAL

One-day-old White Leghorn chicks were fed the vitamin B_{12}-deficient basal diet described by Spivey Fox et al. (9). Control chickens were given weekly injections of vitamin B_{12} at a dosage of 3 μg/chick for the first week and 9 μg/chick thereafter. After 4 to 8 weeks the chickens were killed and their livers removed and made into acetone powders.

Methylene tetrahydrofolic dehydrogenase was obtained by purification from the acetone powders by the procedure described by Huennekens et al. (10) and Hatefi et al. (11) through the step in which the enzyme is eluted from the calcium phosphate gel. This eluate was assayed for methylene tetrahydrofolic dehydrogenase to obtain the data given in tables 1 and 2.

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TABLE 1

Effect of vitamin B_{12} in vivo on chick liver methylene tetrahydrofolic dehydrogenase[1,2]

Exp. no.	Enzyme activity[3]	
	Control	Vitamin B_{12}-deficient
	units/mg protein	*units/mg protein*
1	56	32
2	275	223
3	1230	233
4	1080	414
5	251	160

[1] The contents of the cuvettes were: 0.2 ml enzyme preparation; 150 μmoles phosphate buffer, pH 7.5; 0.48 μmoles TPNH; and 0.68 μmoles of anhydroleucovorin (N^5,N^{10}-methenyl tetrahydrofolic acid). TPNH was omitted from the blank. Final volume, 3 ml; temperature, 25°C.

[2] In each experiment the enzyme preparations were made from one control and one deficient chick liver under as nearly identical conditions as possible. Different birds were used in each experiment.

[3] The units of enzyme activity equal the change in optical density at 340 mμ × 1000/minute.

TABLE 2

Vitamin B_{12} content and methylene tetrahydrofolic dehydrogenase activity in chick livers[1,2]

Liver no.	Dietary supplement	Vitamin B_{12} content	Enzyme activity
		mμg/mg protein	*units/mg protein*
1	None	0.62	233
2	None	3.58	414
3	Vitamin B_{12}	5.22	1080
4	Vitamin B_{12}	7.45	1230

[1] The conditions of the assay are the same as those described in table 1.

[2] The results were obtained on individual chicks.

For the studies of the effects of vitamin B_{12} and coenzyme B_{12} in vitro the enzyme was further purified by the addition of cold saturated ammonium sulfate, pH 8.0, to the eluate to make a 55% saturated solution. The precipitate was taken up in 0.1 M phosphate buffer, pH 7.5, and used as the enzyme.

For the preparation of the enzyme from bone marrow the marrow was homogenized with 10 volumes of 0.1 M phosphate buffer, pH 6.5, in an all-glass homogenizer. The homogenate was shaken with activated charcoal[6] for 2 minutes. This step appeared to remove endogenous nucleotides and to permit greater precision in the assay on this relatively crude material. The activated charcoal was separated by centrifugation and the supernatant solution diluted with 10 volumes of 0.1 M phosphate buffer, pH 7.5; 0.2 ml of this solution was used as the enzyme source. Twelve chicks from each group were used in these experiments.

Methylene tetrahydrofolic dehydrogenase was assayed spectrophotometrically. The content of the cells is given in table 3 for the assays on bone marrow and in table 1 for the assays on liver preparations. In both instances the blank consisted of the reaction mixture less TPNH. Since our enzyme preparations contained large amounts of cyclohydrolase, it was not possible to use the usual blank of omission of anhydroleucovorin (N^5, N^{10}-methenyl tetrahydrofolic acid). The action of cyclohydrolase on anhydroleucovorin leads to the formation of N^{10}-formyl-tetrahydrofolic acid and the net effect of this reaction would also be a reduction in optical density at 340 mμ. The non-enzymatic degradation of the anhydroleucovorin to N^{10}-formyl-tetrahydrofolic acid and its oxidation products in the presence of the phosphate buffer is also taken into account and corrected for by the inclusion of the substrate in the blank. The unit of enzyme activity used is defined in table 1.

The anhydroleucovorin was made by mixing equal volumes of 0.3 M hydro-

[6] Norit, Pfanstiehl Laboratories, Waukegan, Illinois.

TABLE 3

Effect of vitamin B_{12} in vivo on chick bone marrow methylene tetrahydrofolic dehydrogenase[1]

Without anhydroleucovorin		With anhydroleucovorin	
Vitamin B_{12}-injected	Vitamin B_{12}-deficient	Vitamin B_{12}-injected	Vitamin B_{12}-deficient
10.00	9.5	82.1 ± 6.7[2]	57.8 ± 2.5[2]

[1] The contents of the cuvettes were: 2 mg wet weight of bone marrow, 2 μmoles KCN, 150 μmoles of phosphate buffer, pH 7.5, 0.55 μmoles of TPNH and 0.1 μmoles of anhydroleucovorin (N^5,N^{10}-methenyl tetrahydrofolic acid). TPNH was omitted from the blank. In certain cuvettes anhydroleucovorin was omitted. The results are reported as decrease in optical density at 340 mμ × 1000 in 10 minutes. Final volume was 3 ml, the reaction was run at room temperature. The figures given are the average of determinations made on 12 chicks in each group.

[2] SE of mean.

chloric acid containing 1 mg/ml ascorbic acid and a 5.0×10^{-3} M solution of N^5-formyl-tetrahydrofolic acid[7] in an evacuated tube and allowing it to stand 3 to 4 hours. The amount of N^5, N^{10}-methenyl tetrahydrofolic acid formed was estimated from its absorption at 365 mμ (12).

Vitamin B_{12} and coenzyme B_{12} were in crystalline form.[8] The vitamin B_{12} content of the enzyme preparations was determined by a standard microbiological assay using *L. leichmannii* (13).

To determine the physiological significance of the vitamin B_{12} effects upon methylene tetrahydrofolic dehydrogenase, 3- to 4-month-old White Leghorn chicks obtained in the manner described earlier were injected intraperitoneally with tracer amounts of formate-C^{14} or formaldehyde-C^{14}. After 4 hours the chicks were killed and the bone marrow from both femurs removed and stirred into 5 ml of 0.4 N perchloric acid at 0°C. The extraction and precipitation of the nucleic acids and their hydrolysis to the purine and pyrimidine bases and the separation of the bases was carried out as described by Mukherjee and Heidelberger (14) with the following modification. After separation of the bases on paper the spots were eluted from the paper with alcoholic ammonia (73:24:3 ethanol: water:NH_4OH) and the absorption at 250 mμ, 260 mμ and at the wavelength of maximal absorption for each base was read on a Zeiss spectrophotometer. The readings were made against a blank consisting of the eluate from a spot of the same size as the sample cut from the paper at the level of the sample. The identity and purity of the bases was determined by their R_f values on the paper chromatography and by the ratio of absorption at 280/260 mμ and at 250/260 mμ. The concentration of the bases present was calculated from their molar absorbancy indices at the wavelength of maximal absorption as measured by standards dissolved in the eluate described and in agreement with previously observed values (15). An aliquot of each sample was then evaporated in a planchet for counting in a windowless flow counter.

RESULTS

Under our conditions of assay, bone marrow from vitamin B_{12}-injected chicks exhibited significantly higher methylene tetrahydrofolic dehydrogenase activity than did bone marrow from vitamin B_{12}-deficient chicks (table 3). Similar results were obtained with the partially purified liver preparations as indicated in table 1. In each of the 5 experiments, livers from vitamin B_{12}-deficient chicks exhibited less enzyme activity than did livers from vitamin B_{12}-injected chicks. In one experiment 4 chick livers, 2 from the control group and 2 from the deficient group, were compared for vitamin B_{12} content and for methylene tetrahydro folic dehydrogenase activity (table 2). Enzyme activity appeared to be correlated with vitamin B_{12} content. The vitamin B_{12}-deficient chicks used in these experiments were fed the deficient diet for 4 weeks.

To test the in vitro effect of vitamin B_{12} on methylene tetrahydrofolic dehydrogenase a further purification was made by precipitation of the enzyme in 55% saturated ammonium sulfate, pH 8.0. Enzyme preparations from livers of control chicks showed little or no response to vitamin B_{12}. Since these preparations contained endogenous vitamin B_{12} which could mask a vitamin B_{12} requirement, a means of removing the vitamin B_{12} was investigated. Acid precipitation in ammonium sulfate according to the method of Ochoa and coworkers (16) failed to reduce the amount of vitamin B_{12}/mg protein.

When livers from chicks that had been fed the deficient diet for the full 8 weeks were used as the source of material for the enzyme purification, it was not necessary to attempt any resolution of the enzyme to obtain an in vitro stimulation of the enzyme by vitamin B_{12} or by coenzyme B_{12}. The enzyme preparation itself, when carried through the precipitation in basic ammonium sulfate, contained less than 1.0 mμg vitamin B_{12}/mg protein. Since approximately 0.4 mg protein in 3 ml was used as the enzyme in the assays, the amount of endogenous vitamin B_{12} present in the enzyme during the assays was of the order of 1×10^{-10} M or less. Such en-

[7] Obtained commercially from Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

[8] Vitamin B_{12} was obtained commercially from Nutritional Biochemicals Corporation, Cleveland, Ohio. Coenzyme B_{12} was generously provided by Dr. Karl Folkers of Merck and Company, Rahway, New Jersey.

zyme preparations exhibited little or no activity without the addition of exogenous vitamin B_{12} or coenzyme B_{12} (fig. 1). The K_m for the stimulation of the enzyme by both vitamin B_{12} and coenzyme B_{12} was 2.9×10^{-10} M. The stimulation by the vitamin in addition to the coenzyme is in contrast to the results in our earlier experiments.[9] In retrospect it appears that the importance of the sequence of addition of the vitamin B_{12} to the assay (as reported in fig. 1), which had not been observed at that time, was the cause of the original lack of stimulation.

Because of the report by Peel (17) of the indirect effect of vitamin B_{12} and some of its derivatives in the enzyme system studied by Rabinowitz (18), it was decided to test the effect of vitamin B_{12} in an anaerobic assay for methylene tetrahydrofolic dehydrogenase. No stimulation of the enzyme by either the vitamin or the coenzyme form was observed when the assays were run under nitrogen.

This lack of stimulation of the enzyme under anaerobic conditions and the lack of specificity between the vitamin and the coenzyme form of vitamin B_{12} made it imperative to test the physiological significance of these in vitro observations. When formate-C^{14} or formaldehyde-C^{14} was injected into control and vitamin B_{12}-deficient chicks, the specific activity of both purines and of thymine was diminished in the deficient tissues (table 4). The percentage

[9] See footnote 4.

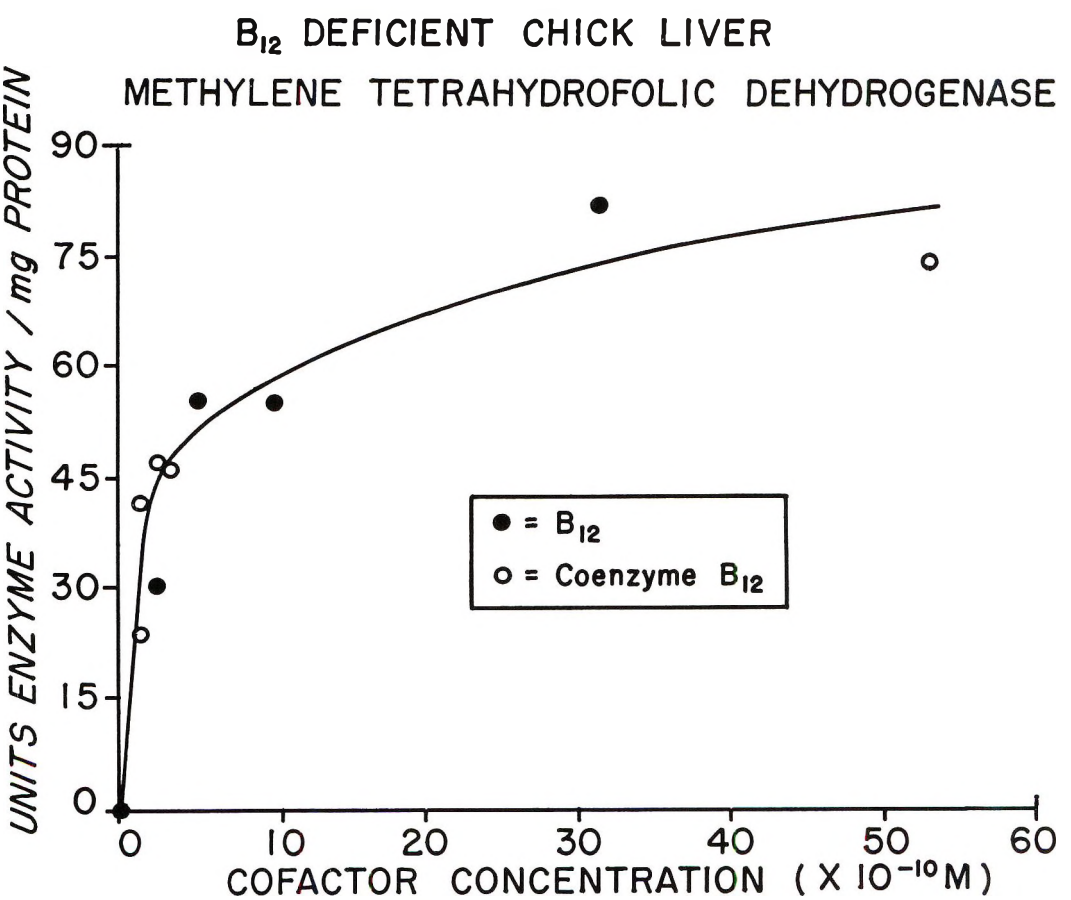

Fig. 1 The response of partially purified, vitamin B_{12}-deficient chick liver preparations of methylene tetrahydrofolic dehydrogenase to additions of vitamin B_{12} or coenzyme B_{12} in vitro. Assay conditions as described in table 1. Both the vitamin and the coenzyme must be added to the enzyme preparation before the substrate to obtain stimulation.

TABLE 4

Effect of dietary vitamin B_{12} on uptake of one-carbon units into nucleic acids in chick bone marrow[1]

Tracer	Dietary supplement	Specific activity			Thymine specific activity / Purine specific activity ratio
		Thymine	Adenine	Guanine	
		count/min/ μmole	*count/min/ μmole*	*count/min/ μmole*	
Formate-C^{14}	0	667	1957	1770	0.36 ± 0.04[2]
Formate-C^{14}	vitamin B_{12}	1013	2437	2339	0.41 ± 0.02[2]
% decrease in deficient tissue		34%	20%	24%	12%
Formaldehyde-C^{14}	0	740	1843	1740	0.42 ± 0.02[2]
Formaldehyde-C^{14}	vitamin B_{12}	1131	2244	1823	0.56 ± 0.02[2]
% decrease in deficient tissue		35%	18%	5%	25%

[1] Each figure is the average of the results obtained from 6 control or 6 deficient chickens for the formate data and from 3 control or 3 deficient chickens for the formaldehyde data. The chicks were injected with 10 μc/100 g body weight formate-C^{14} (3.4 mc/mmole) or 6.7 μc/100 g body weight formaldehyde-C^{14} (4.1 mc/mmole). To minimize any variation that may have occurred in the amount of tracer injected and absorbed in the individual birds, the final results for each chick were calculated in terms of the ratio of the thymine specific activity to the purine specific activity and the average of the ratios for each group is given here. The degree of the deficiency in the chickns, as determined by a microbial assay (15), indicated an average of 57 mμ gm vitamin B_{12}/g kidney in the deficient birds as compared with 1540 mμ g vitamin B_{12}/g kidney in the controls.

[2] SE of mean.

decrease in the specific activity of thymine was greater than that in the purines. The number of chickens used in the experiment was small and the final results, calculated in terms of the ratio of thymine specific activity to purine specific activity, indicate a statistically significant difference between the control and the deficient data only in the case of the formaldehyde tracer ($P = 0.01$) and not for the formate tracer. However, the results with both tracers are similar in degree and direction. There is no greater inhibition of formate over formaldehyde uptake into thymine as would be expected if vitamin B_{12} were a cofactor for methylene tetrahydrofolic dehydrogenase.

DISCUSSION

Figure 2 presents current concepts of the metabolic transformations of one-carbon compounds. This formulation was taken from reports in the literature (7, 19). Despite the data presented here and in earlier reports from our laboratory (1–3, 20) and other laboratories (4–6) which indicate that vitamin B_{12} operates between the formate and formaldehyde levels of oxidation, a dietary deficiency of vitamin B_{12} in chickens does not affect differentially the uptake of formate and formaldehyde into either thymine or purines. The deficiency does diminish the use of these precursors for thymine biogenesis to a greater extent than for purine biogenesis. This can be interpreted in 2 ways. Either vitamin B_{12} plays a significant role in thymine methyl biogenesis at a step beyond the formaldehyde level of oxidation or DNA synthesis has been impaired because of an inability to form deoxyribose as has been observed in microorganisms (21,22) and this has indirectly led to a decreased formation of thymine. The report of Bolinder and Reichard (25) in which vitamin B_{12}-deficient chick embryos showed no decrease in uptake of cytidine into RNA or DNA-deoxycytidine, but did show a decreased uptake of cytidine and deoxyuridine into DNA thymidine, strongly supports the interpretation of the present data in terms of a role of vitamin B_{12} in thymine methyl biogenesis. Regardless of the exact site of action, these results clearly demonstrate a role of vitamin B_{12} in DNA biosynthesis, a function compatible with the hematopoietic role of the vitamin.

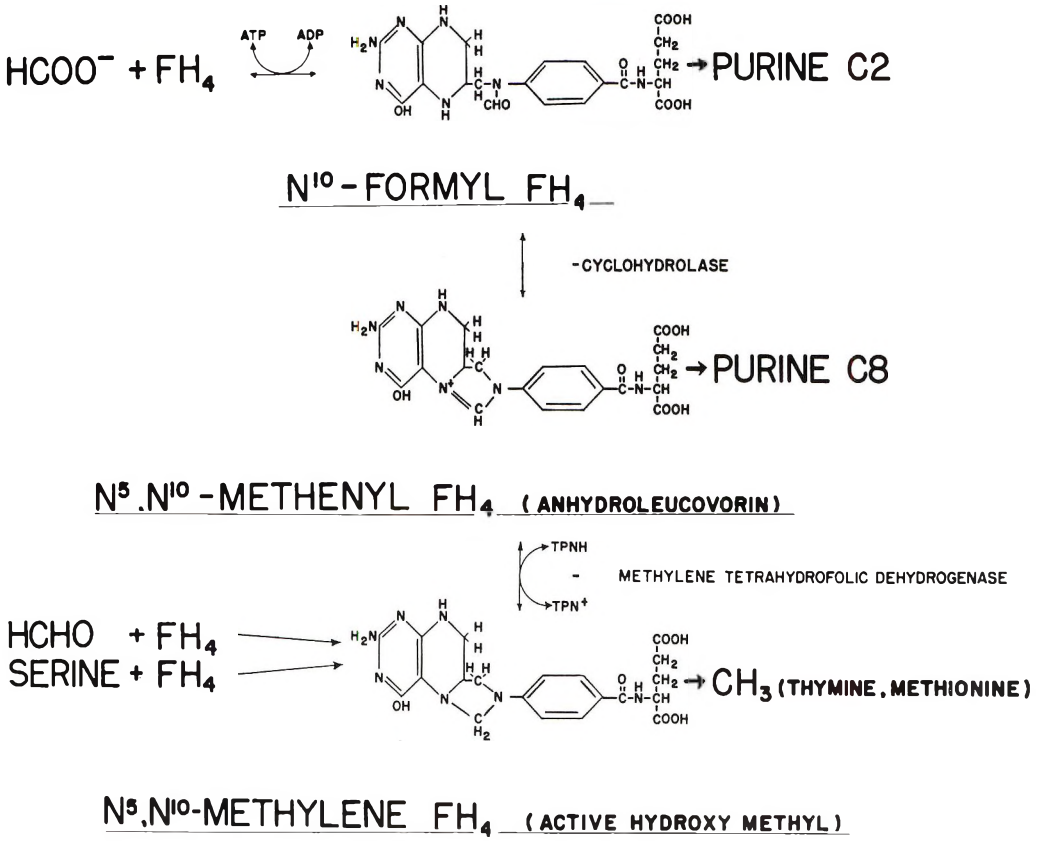

Fig. 2 Current concepts of metabolism of one-carbon compounds. (FH_4 is tetrahydrofolic acid.)

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Effect of Delayed Supplementation of Wheat Gluten with Lysine and Threonine on its Capacity to Promote Growth in the Weanling Rat

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ABSTRACT When the food intake of rats was restricted, lysine supplementation of a wheat gluten diet was equally effective in supporting growth whether it was incorporated into the diet or given by gavage 6 hours after feeding. When food intake was unrestricted, incorporation of lysine into the diet proved to be the more effective mode of administration. If the second limiting amino acid, threonine, was also supplied, with consequent more rapid growth, incorporation of lysine into the diet became more effective even if feeding was restricted. The growth response to threonine supplementation of a wheat gluten-lysine diet was found to be independent of the time of supplementation.

Berg and Rose (1), using a tryptophan deficient diet, were the first to show that amino acid supplementation of a diet lacking an amino acid may be relatively ineffective if the amino acid is not administered approximately simultaneously with the diet. Since that time the results with tryptophan have been confirmed and the same principle has been found to apply to diets deficient in lysine and methionine (2). It has been generally accepted that this principle applies to all incomplete amino acid mixtures or proteins, i.e., supplementation with any amino acid is relatively ineffective unless the supplement is ingested at approximately the same time as the deficient amino acid mixture or protein. Furthermore, it has been assumed that this is so because the animal's body is unable to store excess amino acids but catabolizes them immediately. More recently, Yang et al. (3) reported that lysine, when administered by intubation as much as 16 hours after feeding a wheat flour-based diet, was equally as effective in increasing growth rate and food efficiency as when it was incorporated into the diet. These experiments, however, were carried out under conditions of restricted food intake.

Reported herein are the results of studies designed to determine the relative effectiveness of supplementation of diets deficient in lysine or threonine by incorporation of the limiting amino acid into the diet, or by administering it apart from the bulk of the diet by gavage under conditions of restricted and unrestricted food intake.

EXPERIMENTAL AND RESULTS

Male weanling rats of the Holtzman strain weighing 60 to 70 g were housed individually in wire-bottom cages in quarters maintained at constant temperature and humidity. Ten animals were used on each dietary regimen and all were allowed free access to water.

The protein of the diets was supplied by wheat gluten of which lysine and threonine are the first and second limiting amino acids, respectively. The basal diet contained 1.6% nitrogen and had the following percentage composition: wheat gluten,[1] 17; salt mixture, (4) 4; cellulose,[2] 5; corn oil,[3] 8; glucose, 66 and a complete vitamin addendum.[4]

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[1] Vicrum, Huron Milling Company, Huron, Michigan.

[2] Cellu Flour, Chicago Dietetic Supply House, Chicago.

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

[4] Micronutrients/100 g of food: (in milligrams) thiamine·HCl, 1.0; riboflavin, 2.0; pyridoxine·HCl, 1.0; Ca pantothenate, 10.0; niacinamide, 10.0; inositol, 5.0; choline, 100.0; p-aminobenzoic acid, 30.0; biotin, 0.05; folic acid, 0.2; α-tocopherol, 14.2; menadione, 14.2; vitamin B_{12} triturate (0.1% triturate with mannitol), 10.0. In units: ergocalciferol, 300; vitamin A palmitate, 1600.

The experimental diets are shown in table 1. All additions were made at the expense of glucose. In the first experiments, rats of groups 1 to 3 were allowed free access to diets A (wheat gluten), B (wheat gluten + 0.3% L-lysine·HCl), and E (wheat gluten + 0.6% lysine + 0.45% DL-threonine), respectively. Food consumption was measured daily for animals receiving diets B and E. Groups 4 and 5 were allowed to consume diets A and C

TABLE 1

Composition of experimental diets

Diet	
A	Basal
B	Basal + 0.3% L-lysine·HCl
C	Basal + 0.6% L-lysine·HCl
D	Basal + 0.45% DL-threonine
E	Basal + 0.6% L-lysine·HCl + 0.45% DL-threonine

TABLE 2

Effect of feeding rats lysine- and threonine-deficient diets with the amino acid supplements either mixed in the diet or administered apart from the diet

Group no.	Dietary treatment	28-Day wt gain	Body length	Wt of fat body	Fat body
		g	*cm*	*mg*	*% body wt*
1	Diet A, (wheat gluten) ad libitum	31.6 ± 1.5[1]	15.90 ± 0.17	501 ± 36	0.58
2	Diet B, (wheat gluten + 0.3% L-lysine·HCl) ad libitum	85.6 ± 6.2	18.66 ± 0.14	1359 ± 52	0.90
3	Diet E, (wheat gluten + 0.6% L-lysine·HCl + 0.45% DL-threonine) ad libitum	146.5 ± 5.4	20.13 ± 0.15	1918 ± 108	0.91
4	Diet A, (wheat gluten) ad libitum + L-lysine·HCl by gavage	65.7 ± 3.7	17.67 ± 0.24	899 ± 35	0.72
5	Diet C, (wheat gluten + 0.6% L-lysine·HCl) ad libitum + DL-threonine by gavage	138.4 ± 7.2	20.01 ± 0.18	1722 ± 123	0.85
6	Diet A, (wheat gluten), 10 g/day	13.6 ± 1.5			
7	Diet B, (wheat gluten + 0.3% L-lysine·HCl), 10 g/day	48.9 ± 1.9	17.8 ± 0.08	870 ± 35	0.76
8	Diet E, (wheat gluten + 0.6% L-lysine·HCl + 0.45% DL-threonine), 10 g/day	67.8 ± 1.7	18.3 ± 0.09	710 ± 57	0.59
9	Diet A, (wheat gluten), 10 g/day + 30 mg L-lysine·HCl by gavage	44.5 ± 1.8	17.4 ± 0.10	790 ± 52	0.72
10	Diet C, (wheat gluten + 0.6% L-lysine·HCl), 10 g/day + 45 mg DL-threonine by gavage	68.0 ± 1.9	17.9 ± 0.10	820 ± 45	0.62
11	Diet E, (wheat gluten + 0.6% L-lysine·HCl + 0.45% DL-threonine) ad libitum	126.3 ± 3.5	19.2 ± 0.19	1440 ± 100	0.75
12	Diet D, (wheat gluten + 0.45% DL-threonine) ad libitum + L-lysine·HCl by gavage	79.2 ± 3.9	18.0 ± 0.21	1010 ± 72	0.70
13	Diet E, (wheat gluten + 0.6% L-lysine·HCl + 0.45% DL-threonine), 10 g/day	65.0 ± 1.8	17.1 ± 0.17	870 ± 39	0.67
14	Diet D, (wheat gluten + 0.45% DL-threonine), 10 g/day + 60 mg L-lysine·HCl by gavage	47.7 ± 1.8	16.7 ± 0.11	660 ± 25	0.59

[1] SE.

(wheat gluten + 0.6% lysine·HCl) ad libitum and in addition each was given by stomach tube in a single dose 6 hours after feeding, the quantity of L-lysine·HCl or DL-threonine calculated to have been consumed on the previous day by the animals receiving diets B and E, respectively.

The rats comprising groups 6 to 10 were each restricted to 10 g of food daily as follows: group 6, diet A; group 7, diet B; group 8, diet E; group 9, diet A + 30 mg L-lysine·HCl by stomach tube 6 hours after feeding; and group 10, diet C + 45 mg DL-threonine by stomach tube 6 hours after feeding.

The animals were weighed at 5-day intervals and were killed after 28 days, when body length was measured and the epididymal fat bodies were weighed. The results shown in table 2 are the averages of at least 2 experimental groups.

The listed averages of body weight gains, body lengths and weights of epididymal fat bodies of rats of group 7 are not significantly different from the corresponding values for animals of group 9. This observation corroborates that of Yang et al. (3) that the time of supplementation of a wheat protein based diet with its first limiting amino acid, lysine, is not critical when the diet is restricted. The averages of body weight gains, body lengths and weights of fat bodies of animals of group 4, however, are all significantly less than the corresponding values for animals of group 2 ($P < 0.01$). This result demonstrates clearly that for maximal growth when food is unrestricted the time of supplementation is critical. Unexpectedly, the growth response due to the second limiting amino acid, threonine, was independent of the time of supplementation whether feeding was restricted or ad libitum. (The listed averages of the values for groups 3 and 5 and for groups 8 and 10 are not significantly different.)

It has been demonstrated that total body fat is proportional to the weight of the epididymal fat body (5). The weights of these organs and the body length measurements indicate that the observed differences in weight were not due solely to differences in amount of fat deposited.

To test the possibility that time of lysine supplementation might be critical even with restricted food intake if the protein were of a higher quality and the animals, therefore, growing more rapidly, the following experiment was performed. The animals of group 11 were allowed unrestricted access to diet E. The food consumed was measured daily. Group 12 was allowed to consume diet D (basal + 0.45% DL-threonine) ad libitum and in addition each animal was given by stomach tube in a single dose the quantity of L-lysine·HCl calculated to have been consumed on the previous day by the animals receiving diet E. The rats in group 13 and 14 were each restricted to 10 g of diet E and D, respectively. In addition, group 14 received by stomach tube 6 hours after feeding 60 mg of L-lysine·HCl. The 28-day average weight gains of rats of duplicate experiments are also recorded in table 2. The listed averages of body weight gains, body lengths and weights of fat bodies of animals of group 13 are all significantly greater than the corresponding values for animals of group 14 ($P < 0.02$), and they show that rats even with a restricted food intake will utilize lysine more efficiently when it is incorporated into the diet than when given by stomach tube in a single daily dose if the quality of the protein is sufficiently high.

DISCUSSION

It has been adequately demonstrated that the lysine requirement for maintenance is much lower than that for growth (6, 7). This fact may help to explain why time of administration of lysine to rats ingesting a lysine-deficient diet, deficient also in a second amino acid, is not critical, when food is restricted and why, when the second limiting amino acid is also supplied with consequent more rapid growth it becomes so.

The results obtained in the present study with threonine supplementation make untenable the hypothesis that for efficient utilization all essential amino acids must be administered approximately simultaneously. These results also raise the question of the storage of threonine in the animal body. It is a matter of interest whether threonine is unique in this respect or whether one or more of those amino acids yet untested — histidine, leucine,

phenylalanine and valine — may also be well utilized by the rat when fed at a different time than the remainder of the diet. The report by Spolter and Harper (8) that injected isoleucine is much less effective than injected lysine in supporting growth of rats suggests that delayed supplementation with isoleucine would be relatively ineffective.

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Action of Vitamin A on Liver Homogenate Oxidation of Tricarboxylic Acid Cycle Intermediates[1]

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ABSTRACT Vitamin A deficiency in young male albino rats results in a markedly elevated rate of oxidation of pyruvate, citrate, α-ketoglutarate, succinate, glutamate and fumarate, but not β-hydroxybutyrate and caprylate by liver homogenates. This effect was not observed with kidney or heart preparations and it was not due to reduced food consumption. The administration of vitamin A to deficient animals reduced the oxidation rates to normal values within 48 hours.

The role of vitamin A in the visual cycle has been elucidated in great detail primarily through the efforts of Wald and his staff (1, 2). However, it is evident that the action of vitamin A in higher animals is not accountable solely on the basis of one such system. This is especially emphasized by the demonstration that vitamin A acid can replace vitamin A alcohol in correcting deficiency lesions except the defect in the visual cycle (3). In other work, Fell and associates (4) have shown that excess vitamin A results in the resorption of cartilage rudiment organ cultures presumably by the release of hydrolytic enzyme from the cells (5). An additional action of vitamin A on mucopolysaccharide biosynthesis in vitro has been described by Wolf and collaborators (6, 7).[3] However, a unified concept of the mechanism of vitamin A action has not yet evolved.

A possible role of vitamin A in respiratory systems was suggested by several sources. An increased basal metabolic rate in vitamin A-deficient animals has been reported from different laboratories (8–10). In in vitro studies, Redfearn (11) noted an increase in endogenous oxidation by liver homogenates from vitamin A-deficient rats. More recently, Vignais (12) observed an increase in the NADPH cytochrome c reductase and a decrease in the pyridine nucleotide transhydrogenase activities of liver mitochondria from vitamin A-deficient rats. He suggested that this might result in respiration uncoupled from phosphorylation and might account for the observed elevation in basal metabolic rate. Ray and Sadhu (13) recently reported that administration of excess vitamin A to rats resulted in a significantly decreased oxidation of succinate by liver slices and homogenates. Wolf et al., on the other hand, in a few experiments reported no change in oxidative phosphorylation or in $C^{14}O_2$ production from injected radioactive acetate due to vitamin A deficiency (14, 15). The question of a possible role of vitamin A in respiratory systems was therefore reopened. In the present paper, results are reported which demonstrate a marked increase in the oxidation of tricarboxylic acid cycle intermediates by homogenates of liver from vitamin A -deficient rats.

METHODS

Male weanling rats[4] were housed in overhanging wire cages. They were given food and water ad libitum throughout unless otherwise indicated. The diet was a modification of that used by Bellin and Steenbock (16) and consisted of: (in per cent) vitamin-free casein,[5] 18; cystine, 0.2; salts (calcium and phosphorus free), 2; $CaCO_3$, 1.0; an equimolar mixture of K_2HPO_4 and KH_2PO_4 to give 0.3% P in the diet; roughage,[6] 3; cottonseed oil,[7] 10; vitamins and

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[2] National Science Foundation Fellow.

[3] Wolf, G., and A. Moretti 1961 Vitamin A and net synthesis of mucopolysaccharides. Federation Proc., 20: 162 (abstract).

[4] Obtained from the Holtzman Company, Madison, Wisconsin.

[5] "Vitamin-Free" Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[6] Cellu Flour, Chicago Dietetic Supply House, Chicago.

[7] Wesson Oil, Wesson Sales Company, Fullerton, California.

glucose monohydrate,[8] 65. The calcium and phosphorus free salts contained: (in per cent) KCl, 57.5; NaCl, 20.9; $MgSO_4$, 17.9; $FeSO_4 \cdot 7H_2O$, 3.22; NaF, 0.113; $CuSO_4$, 0.078; $MnSO_4$, 0.04; $K_2Al_2(SO_4)_2$, 0.018; KI, 0.010; $CoCl_2 \cdot 6H_2O$, 0.004; and $Na_3AsO_4 \cdot 12H_2O$, 0.0017. The water-soluble vitamins were added to give the following: (in mg/kg of diet) Thiamine, 5; riboflavin, 5; pyridoxine, 5; vitamin B_{12}, 0.02; Ca pantothenate, 28; nicotinamide, 20; inositol, 200; folic acid, 0.2; biotin, 0.1; and choline chloride, 500. A supplement of fat-soluble vitamins in cottonseed oil[9] supplied 875 μg α-tocopherol, 105 μg 2 methyl, 1,4-naphthoquinone, and 225 IU of vitamin D_2/week.

Unless otherwise noted, the rats were divided equally into 2 groups. One group received no vitamin A, whereas each rat in the remaining group received either 33 IU vitamin A acetate or its equivalent of β-carotene in cottonseed oil[10] daily. The rats became severely deficient in vitamin A after having received the unsupplemented diet for 28 to 35 days, as indicated by xerophthalmia, weight loss and in some cases ataxia. Upon the appearance of these symptoms, the animals were killed by a sharp blow on the head followed by partial decapitation. The tissues were removed quickly, chilled and then homogenized in 0.154 M KCl or 0.25 M sucrose at 0°C to yield a 10% w/v homogenate by means of a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The homogenates (0.3 to 0.5 ml) were added to chilled Warburg vessels containing a solution of 3 μmoles ATP, 40 μmoles potassium phosphate buffer, pH 7.3, 10 μmoles $MgCl_2$, substrate, 0.08 μmoles cytochrome c, and either 335 μmoles sucrose or 280 μmoles KCl in 3 ml final incubation volume. The concentration of substrates was 15 μmoles/vessel except succinate (30 μmoles) and citrate (45 μmoles). When pyruvate or caprylate were used as substrates, 0.1 μmole of fumarate was added as the 4-carbon primer. Oxidation uptake was measured for 30 minutes at 37°C by standard Warburg techniques. The center well contained 0.2 ml 10% KOH absorbed on filter paper and the gas phase was air. An aliquot of each homogenate was analyzed for N by Nesslerization following digestion by a slight modification of the method of Johnson (17). The results are expressed as microliters of oxygen consumed per hour per milligram of homogenate nitrogen ($Q_{O_2}N$).

RESULTS

In the first series of experiments, the oxidation of a variety of substrates by liver KCl homogenates from vitamin A-deficient and vitamin A fed-rats was investigated. Quite clearly, vitamin A deficiency markedly increased the oxidation rates of pyruvate, citrate, α-ketoglutarate, glutamate, succinate, and fumarate (table 1). No effect was observed with either β-hydroxybutyrate or caprylate. These results suggest that the action of vitamin A is on a system common to all the tricarboxylic acid cycle substrates such as electron transport, oxidative phosphorylation or mitochondrial structure. However, on this basis it is difficult to provide an explanation for the lack of effect with β-hydroxybutyrate or caprylate, although with the latter, the explanation may be related to its surface active properties.

TABLE 1

Effect of vitamin A on the oxidation of substrates by liver homogenates

Substrate	Without vitamin A	With vitamin A[1]	Decrease
	$Q_{O_2}(N)$	$Q_{O_2}(N)$	%
Pyruvate[2]	109 ± 4.7[3]	85 ± 2.3[3]	22
Citrate	144 ± 4.4	97 ± 5.0	33
α-Ketoglutarate	154 ± 5.9	102 ± 4.0	34
Glutamate	149 ± 9.2	106 ± 5.7	30
Succinate	205 ± 7.5	152 ± 5.9	26
Fumarate	130 ± 6.64	94 ± 2.4	28
β-OH-Butyrate[2]	96 ± 3.9	90 ± 3.7	6
Caprylate[2]	132 ± 5.6	144 ± 5.4	0

[1] Each rat received the equivalent of 33 IU of vitamin A daily.
[2] 3×10^{-4} M fumarate was present in these vessels to provide oxalacetate primer.
[3] SE of mean was calculated from at least 6 determinations.

The effect of vitamin A on homogenate oxidations was not observed with kidney or heart, whereas in the same animals a striking effect was noted with liver (table 2).

[8] Cerelose, Corn Products Company, Chicago 6, Illinois.
[9] See footnote 7.
[10] See footnote 7.

TABLE 2

Lack of vitamin A effect on the oxidation of citrate, succinate and glutamate by homogenates of kidney and heart[1]

Tissue	Vitamin A	Citrate	Succinate	Glutamate
		Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)
Liver	—	139	187	161
	+	94	138	108
Kidney	—	198	485	267
	+	223	474	245
Heart	—	—	358	389
	+	—	365	412

[1] There were at least 4 rats in each group, and tissues from the same rats were used for all determinations. Conditions as described in the text.

From these results it appeared clear that liver was the organ of the choice in further experiments. However, this does not necessarily mean that vitamin A is without a basically similar action in the other organs since oxygen consumption is governed by many factors which may differ or have differing influence in the various organs.

Paired feeding of vitamin A supplemented rats and deficient rats resulted in virtually the same results as with ad libitum-fed rats (table 3). Similarly in a parallel experiment, it could be shown that the restriction of food to stock rats had little or no effect on their liver oxidation of citrate and glutamate (table 3). Furthermore, when vitamin A was given to deficient rats and the food was restricted to the deficiency level, the oxidation rates nevertheless decreased to normal values (table 4). It was, therefore, concluded that the increase in liver homogenate oxidations due to vitamin A deficiency was not the result of reduced food consumption.

Finally, it appeared important to establish whether this lesion in the liver could be reversed by the administration of vitamin A and if so, how rapidly this would occur. Vitamin A-deficient rats were divided equally into 2 groups. One group received a single dose of vitamin A acetate (3000 IU), whereas the remaining group served as deficient controls. Pairs of deficient and supplemented rats were killed at various times following the administration of vitamin A. A group of rats receiving vitamin A throughout were studied simultaneously to serve as positive controls. The results show clearly that not only was the vitamin A able to reverse the lesion but the effect was quite rapid (table 4). Within 48 hours following the administration of the vitamin A, the oxidation rates had decreased to normal values (shown by the rats which had received the vitamin A throughout the experimental period). At 24 hours following administration of the vitamin, perhaps some reduction had occurred with citrate and glutamate but not with succinate. The 48-hour response is one of the most rapid observed thus far for vitamin A given in vivo. For example, its administration restores night vision after only 60 hours have elapsed (18). These results provide additional evidence that the effect of deficiency on homogenate oxidation is not due to secondary effects resulting from severe deficiency.

TABLE 3

Lack of effect of food restrictions on oxidations by rat liver homogenates

Diet	Food restriction	Citrate	Succinate	Glutamate
		Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)
No vitamin A	—	145	213	152
Plus vitamin A	—	96	163	103
Plus vitamin A	+[1]	79	136	103
Stock	—	73	—	125
Stock	+[1]	77	—	122

[1] Rats received 6 g diet/day which is the amount consumed by vitamin A deficient rats at this stage. The rats were maintained with their respective diets for 28 to 34 days. There were at least 6 rats in each group.

TABLE 4

Reduction of vitamin A-deficient liver homogenate oxidations by the administration of vitamin A[1,2]

Hours after vitamin A administration[1]	Citrate	Succinate	Glutamate
	Q_{O_2} (N)	Q_{O_2} (N)	Q_{O_2} (N)
0[3]	137	186	171
6	126	179	170
24	118	180	161
48	90	131	105
72	82	135	90
72[4]	79	136	103
Vitamin A daily throughout	95	138	107

[1] On twenty-eighth day one-half the rats received 3000 IU vitamin A acetate.
[2] Each value represents an average of at least 6 determinations.
[3] The average values from deficient rats killed during the 72-hour period were in close agreement with these values.
[4] Food restricted to 6 g/day/rat.

DISCUSSION

The nature of the changes in homogenate oxidation brought about by the deficiency must remain a matter of speculation. The oxygen consumption was increased with a number of substrates suggesting that the effects of vitamin A deficiency must be on a component or components common to all these systems. However, it is then difficult to explain the lack of effect of the deficiency when caprylate and β-hydroxybutyrate were used as substrates. In view of the work of Dingle and co-workers (19, 20) that vitamin A may be involved in membrane structure and stability, it is possible that the present results are related to changes in mitochondrial structure. Preliminary results with the electron microscope in this laboratory are in agreement with this possibility. If this is the case, then the failure to observe an effect of vitamin A deficiency when caprylate or β-hydroxybutyrate were used as the substrates may well be related to the surface active properties of these compounds. In any case, a thorough examination of the nature of the changes in homogenate and mitochondrial respiratory systems may be of value in the elucidation of the biochemical mechanisms of vitamin A action.

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Effect of Fasting on the Incorporation in Vitro of Palmitate-C^{14} into Glycerolipids of Mitochondria[1]

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ABSTRACT The effect of a 24-hour fast and a re-feeding of glucose, corn oil, or egg albumin by a stomach tube following the fast upon the incorporation of palmitate-1-C^{14} into various lipids of rat liver mitochondria was determined. Both normal animals and animals with regenerating livers were used. Fasting depressed the incorporation of palmitate-1-C^{14} into both triglycerides and total phospholipids. The latter was reflected in an almost complete cessation of the incorporation of C^{14} into ethanolamine phosphatide, and little change in that of the inositol and choline phosphatide. When the fasted rats were fed glucose, the incorporation of C^{14} into triglycerides and ethanolamine and choline phosphatides increased. Re-feeding corn oil depressed the incorporation of palmitate-1-C^{14} into the lipids and re-feeding egg albumin increased C^{14} uptake by the phospholidips but not by the triglycerides. With mitochondria from regenerating livers, the incorporation of palmitate-1-C^{14} into the choline phosphatides was markedly increased, and that in the other lipids decreased when compared with the values found employing mitochondria from normal livers. Fasting the partially hepatectomized animals resulted in lower incorporation of C^{14} into choline phosphatides and triglycerides. Glucose, corn oil, and egg albumin re-feedings generally had the same effects on regenerating liver mitochondria as on normal ones.

Work elsewhere has demonstrated that lipid metabolism may be altered markedly in the fasting animal. For example, there is an increase in serum non-esterified fatty acids and glycerides during fasting (1–3); also a decreased biosynthesis in vitro of fatty acids and cholesterol has been observed to result from fasting (4–9). Lyon et al. (10) reported a decreased conversion of acetate to fatty acids in rats as early as 12 hours after they withheld food. They were able to demonstrate that a lack of dietary carbohydrate was the principal cause of the depressed lipogenesis.

Studies in this laboratory using palmitate-C^{14} in vitro and whole liver homogenate as enzyme have shown that the physiological changes accompanying liver regeneration favor an increased incorporation of palmitate-C^{14} into ethanolamine but not into choline phosphatides. With liver mitochondria as enzyme, however, the incorporation of palmitate-C^{14} into both choline and ethanolamine phosphatide is increased during liver regeneration. In neither case was the formation of triglycerides stimulated appreciably (11).

The present experiments were conducted in an attempt to learn more of the physiological circumstances that might direct the metabolism, selectively, of one or another of the glycerolipids. The incorporation of palmitate-C^{14} into triglycerides, and ethanolamine, choline, and inositol phosphatides in vitro has been studied using normal and regenerating liver tissues obtained from fasted and fasted, re-fed rats.

METHODS

Male Holtzman rats, 3 to 8 months old, weighing 350 to 400 g, were used. They had been reared with a standard commercial pelleted rat diet. Animals were fasted 24 hours as described by Van Bruggen et al. (12). Following a fasting period, the rats were re-fed by stomach tube. Either 5 g glucose in 7 ml water, 2.3 g corn oil, or 5.0 g egg albumin dissolved in 8 ml water was administered in this manner. The animals were killed either at the end of the 24-hour fast, or 6 hours after the re-feeding. When partially hepatectomized animals were used, they were fasted

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for the 24-hours 48 hours following hepatectomy.

The rats were killed and livers perfused as described by Johnson and Albert (13), and mitochondria were prepared according to the method of Stein et al. (14). They were suspended in tris buffered isotonic KCl, and served as enzyme.

The incubation medium contained: (in μmoles) glycerol, 50; magnesium chloride, 50; sodium succinate, 100; glucose, 50; sodium α-glycerophosphate, 25; phosphate buffer pH 7.4, 20; tris buffer pH 7.4, 50; sodium ATP, 5; DPN, 5; coenzyme A, 1; potassium palmitate-1-C^{14} (380,000 count/min), 2.8; and mitochondria equivalent to 2.5 g of fresh liver, in a final volume of 4.5 ml. Incubations were carried out in an atmosphere of air at 37° with shaking for 10 minutes. This incubation period was selected on the basis of earlier experiments which involved incubations up to one hour (11). The reactions were stopped by the addition of 20 ml of methanol. Zero time controls were used in which the reactions were stopped immediately after adding the mitochondria to the media. Lipids were extracted, freed of palmitate-C^{14}, and triglycerides, and ethanolamine, choline, and inositol phosphatides prepared, and radioactivity determined as described previously (11). The identity of the phosphatide fractions was established by us by hydrolyzing each in 1.7 N methanolic·HCl and comparing the hydrolysis products with inositol, and with similarly hydrolyzed phosphoryl ethanolamine and phosphoryl choline, using thin layer chromatography (15).

Phosphorus was determined on all phospholipids by a modification of the method of Fiske and SubbaRow (16), and triglycerides were determined by weighing air-dried aliquots of their solutions.

RESULTS

Data obtained using liver mitochondria from unoperated animals are shown in table 1. Fasting for 24 hours decreased the incorporation of palmitate-C^{14} into both the triglycerides and total phospholipids. The latter reflected a sharp decrease, almost to a complete cessation, of the incorporation of radioactivity into ethanolamine phosphatide. Incorporation into the choline phosphatide fraction was moderately depressed,[3] and no change as a result of fasting was observed in the inositol phosphatide fraction.

When the fasted rats were fed 5 g of glucose by stomach tube, and tissues taken 6 hours later, incorporation of C^{14} into triglyceride was restored to a level exceeding that observed in non-fasted rats. An increased incorporation of C^{14} was observed also in both the ethanolamine phosphatide and choline phosphatide fractions. Again, there was no change in the incorporation of palmitate-C^{14} into inositol phosphatide.

[3] The values for the incorporation of palmitate-1-C^{14} into choline phosphatide of each of the control animals were, respectively, 6760, 7920, 4480, 11080, and 8840 counts/min/mg of lipid.

TABLE 1

Incorporation of palmitate-1-C^{14} into various lipids of rat liver mitochondria

Experimental group	Triglyceride[1]	Total phospholipid[1]	Ethanolamine phosphatide[1]	Inositol phosphatide[1]	Choline phosphatide[1]
Control	5214 (1044–8246)	8480 (5920–11800)	34720 (19320–89320)	12360 (10000–15520)	7120 (4480–11080)
Fasted	2845 (2690–3122)	3660 (3200–3960)	2210 (2080–2360)	11720 (10320–13000)	4970 (4680–5140)
Fasted, glucose-fed	8210 (1528–9272)	5425 (4760–5840)	7760 (5400–12680)	11320 (8480–14240)	6120 (4840–7320)
Fasted, corn oil-fed	1340 (1160–2320)	4065 (3960–4220)	4172 (3040–5120)	6115 (4480–7680)	3320 (3160–3520)
Fasted, egg albumin-fed	2270 (2006–2554)	6365 (5960–6600)	8870 (8615–9280)	11300 (10080–12220)	5635 (5040–6080)

[1] Count/min/mg of lipid. There were 5 animals in the control group, and 3 animals in each of the other groups. Average values are given, with ranges shown in parentheses.

Upon re-feeding corn oil, the incorporation of palmitate-C^{14} remained suppressed in all of the lipids. The incorporation into triglyceride was actually lower than that noted in the fasted animals. The specific activity of ethanolamine phosphatides was approximately twice that observed in the fasted animals, but was low compared with that in non-fasted animals. The incorporation into inositol phosphatide and choline phosphatide was lower than that observed in the fasted animals which were not re-fed.

When the rats were fed egg albumin via stomach tube following the fast, the incorporation of palmitate-C^{14} into triglyceride remained suppressed, but a partial restoration was observed in the total phospholipids. The effect of egg albumin re-feeding was similar to that of glucose, except that the latter resulted in a restoration, to normal levels, of the incorporation of palmitate-1-C^{14} into triglycerides, whereas the former did not.

The results of the experiments using mitochondria from regenerating liver are shown in table 2. The incorporation of palmitate-1-C^{14} into triglycerides was less than that observed when mitochondria from unoperated, non-fasted animals were used. The incorporation into the total phospholipids was approximately the same, although this was not true for individual phospholipids. There was a marked depression of C^{14} uptake into ethanolamine phosphatides, and a probable slight depression in inositol phosphatide. On the other hand, the palmitate-1-C^{14} was incorporated into the choline phosphatide to about twice the extent noted in the non-fasted unoperated controls.

Fasting and re-feeding were, in general, accompanied by similar effects with regenerating livers as with normal ones. When the partially hepatectomized animals were fasted 24 hours there was decreased uptake of palmitate-1-C^{14} into both triglycerides and total phospholipids. Although the incorporation of the isotope into ethanolamine phosphatide fraction of the liver from the fasted animal was slightly higher than that of the non-fasted, it was still much lower than that in the non-fasted unoperated animals. Fasting did not affect the uptake of palmitate-1-C^{14} into inositol phosphatide. The high specific activity of choline phosphatide due to the liver regeneration was depressed as a result of the fast.

Upon re-feeding glucose, the ability of the mitochondria to incorporate palmitate-1-C^{14} into triglyceride was restored, and even exceeded that observed in the non-fasted controls (table 2). The slight increase for the total phospholipids probably reflects the increase observed in the ethanolamine phosphatide fraction. Corn oil re-feeding suppressed the incorporation of palmitate-1-C^{14} into all of the lipids, whereas egg albumin re-feeding had the same

TABLE 2

Incorporation of palmitate-1-C^{14} into various lipids of regenerating rat liver mitochondria

Experimental group	Triglyceride[1]	Total phospholipid[1]	Ethanolamine phosphatide[1]	Inositol phosphatide[1]	Choline phosphatide[1]
Regenerating control	2980 (1762–4256)	8950 (7560–10160)	1320 (1080–1480)	8730 (5760–11240)	14480 (13600–14800)
Fasted	1636 (1470–1738)	5920 (5680–6210)	2360 (2040–2640)	10240 (9880–10520)	6560 (6440–6600)
Fasted, glucose-fed	6234 (5976–6530)	6520 (5300–7640)	8810 (6960–10440)	7820 (7240–8360)	7120 (6520–7640)
Fasted, corn oil-fed	1144 (980–1275)	3040 (2770–3280)	2480 (2000–2600)	4660 (4580–4720)	3180 (2680–3440)
Fasted, egg albumin-fed	2830 (2556–3137)	9020 (8420–9040)	5880 (5840–6250)	10000 (9360–10640)	7440 (7160–7680)

[1] Count/min/mg of lipid. Three animals were used in each group. Average values are given, with ranges shown in parentheses.

effects as the glucose re-feeding, but to a lesser extent.

DISCUSSION

The experiments described here are concerned only with the incorporation of palmitate-1-C^{14} into the glycerolipids indicated, without any assumption being made concerning the pathway of its entry. Also, the data do not permit an interpretation relative to rates of formation of any of the lipids.

Experiments, cited earlier (6), using acetate-C^{14} to determine the effects of fasting on fatty acid synthesis in vivo measure the net formation of the fatty acids plus the incorporation of the latter into the various lipids in which they are found. Although undoubtedly there is some breakdown of the precursor palmitate and its re-incorporation into lipids in a form other than palmitate in the present experiments, this is presumably of minor importance, and the present studies are believed to measure predominantly the incorporation of the palmitate-1-C^{14} intact into the various glycerolipids. Assuming this, the results of the experiments reported here suggest that the depressed incorporation of acetate-C^{14} into fatty acids of phospholipids and neutral lipids following fasting, which has been observed by others (5, 6, 10) is due in part, as least, to a decreased esterification of fatty acid into the lipids. Although it might also be due, in part, to a greater dilution by non-labeled precursor fatty acids present in the tissues, it appears reasonable to suggest, on the basis of the present data, that whether there is actually a depression of the formation of fatty acids from acetate during starvation remains to be demonstrated.

The fact that following a 24-hour fast, the extent of depression of the incorporation of palmitate-1-C^{14} into triglyceride, ethanolamine phosphatide, and choline phosphatide differed widely, is worthy of comment. In view of the similarity in the biosynthetic pathways of ethanolamine and choline phosphatides (17) it was unexpected to find the marked depressing effect of fasting on the incorporation of palmitate-1-C^{14} into ethanolamine phosphatide, and relatively little effect on its incorporation into the choline phosphatide fraction. Preliminary experiments to be reported, in which diglyceride is used as precursor, revealed differences from those reported here, and suggest the present data might reflect metabolic pathways in addition to simple esterification of palmitate-1-C^{14} into the compounds measured.[4] Other investigators recently have described routes of formation for some of the glycerolipids that might be considered in an explanation of the data (18–22).

Inositol phosphatide formation differs from that of ethanolamine or choline phosphatide (23–25) and may account in part for the relative insensitivity of the inositol phosphatides to starvation, under the experimental conditions used in the present work. Of interest, in this respect, is Dittmer and Hanahan's (26) conclusion from studies in vivo that palmitic acid of liver inositides was not derived from the same source as that of other phospholipids.

The present study demonstrates that a short period of fasting appears to alter metabolic activity within the mitochondria of the liver cell, in such a way as partially to explain alterations in lipid metabolism observed with in vivo studies on fasting (12). No inference may be drawn from this study concerning the nature of the change within the mitochondria which results from the fast, however. One possibility is that there is an alteration in mitochondrial enzyme levels or activities, or both. Another is that the fatty acid composition of the mitochondrial phospholipids or glycerides, or both, has been altered as a result of the fast, and this may influence the manner in which any given fatty acid might be incorporated into the lipids of the tissues. Recalling the preferential incorporation of different fatty acids into various lipids observed in vivo by others (26–28), one might expect the present results to vary depending on the nature of the fatty acid employed as precursor. Experiments are underway to investigate this.

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Effect of Soybean Trypsin Inhibitor on Methionine and Cystine Utilization[1]

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ABSTRACT Feeding unheated soybean or heated soybean with a single oral dose of crystalline soybean trypsin inhibitor to rats increases the amount of expired $C^{14}O_2$ derived from a tracer dose of DL-methionine-2-C^{14}. This increased oxidation of labeled methionine is suppressed by feeding supplementary cystine. In growth and protein efficiency studies, cystine supplements unheated soybeans, but has no effect when heated soybeans are fed in an otherwise complete semipurified diet. Unheated soybeans were fed at a level in the diet that would provide the rat's normal requirement for methionine, assuming adequate cystine from the soybean protein (0.17% available methionine). Under these conditions dietary supplementation with either 0.3% L-cystine or 0.3% DL-methionine gave equal growth responses. It is concluded that in the rat, feeding unheated soybeans does not selectively impair the availability or tissue utilization of methionine, but there is a metabolic block in the utilization of cystine for protein synthesis. Furthermore, this block appears to be caused in some unknown manner by the trypsin inhibitors naturally occurring in unheated soybeans.

In spite of years of knowledge that a heat treatment of soybeans is necessary for optimal utilization of the protein, an adequate explanation of the mechanism whereby unheated soybeans exhibit inferior nutritional value has never been given. It has been generally accepted that the trypsin inhibitor in the unheated soybean is the major factor (1). Furthermore, it has been proposed on the basis of indirect approaches that methionine availability or tissue utilization is uniquely depressed when the unheated soybean is fed, presumably an effect of the trypsin inhibitor content (2–4). Evidence has been given that ruled out an impairment of methionine absorption (availability) as an explanation for the low nutritive value of the unheated legume (5, 6). However, a defect in tissue utilization of the amino acid has been given additional support (7). The present investigation was designed to examine, by more direct methods, the influence of unheated soybeans and more specifically the effect of soybean trypsin inhibitors on the tissue utilization of methionine and cystine in rats.

EXPERIMENTAL

In most of the studies reported here, DL-methionine-2-C^{14} was administered to rats by stomach tube or by intraperitoneal injection in a tracer dose of 10 μc/100 g body weight. The rats (Holtzman) were obtained as male weanlings. They were caged individually and pair-fed a diet (table 1) containing either 40% of a well heat-treated, extracted soybean meal or 50% of an extracted, unheated soyflake. It had been determined previously that these relative amounts of the 2 soybean preparations would provide equal quantities of "available" methionine (6). After 2 weeks of this regimen, the rats weighed from 100 to 150 g. Food was removed from the cages and 16 hours later a dose of 50 mg crystalline soybean trypsin inhibitor in 2 ml H_2O (Kunitz type (8)[2] was given by stomach tube to one-half of the rats that had received the heated soybean diet. In all studies either 3 or 4 rats were used for each treatment. Two hours later all of the rats (now divided as 3 groups — unheated soybean, heated soybean, and heated soybean plus trypsin inhibitor) were given DL-methionine-2-C^{14} either by stomach tube or by intraperitoneal injection. Immediately the rats were placed in all-glass, closed metabolism units (9) and

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[2] Nutritional Biochemicals Corporation, Cleveland.

TABLE 1

Composition of soybean diets

Major components	Unheated soybean diet	Heated soybean diet
	%	%
Unheated soyflakes	50.0	—
Heated soybean meal[1]	—	40.0
Glucose monohydrate[2]	27.7	37.7
Salt mixture[3]	4.0	4.0
B vitamins	2.0	2.0
Fat-soluble vitamins	1.0	1.0
Hydrogenated vegetable oil[4]	15.0	15.0
Choline dihydrogen citrate	0.3	0.3
Total	100.0	100.0

B vitamins in 2.0 g glucose monohydrate	
	mg
Thiamine·HCl	0.40
Riboflavin	0.80
Pyridoxine·HCl	0.40
Ca pantothenate	4.00
Niacin	4.00
Inositol	20.00
Biotin	0.02
Folic acid	0.20
Vitamin B_{12}	0.003
Menadione	1.00

Fat-soluble vitamins in 1.0 g corn oil	
	mg
Vitamin A acetate	0.31
Vitamin D (calciferol)	0.0045
α-Tocopherol	5.00

[1] Autoclaved at 107°C for 30 minutes.
[2] Cerelose, Corn Products Company, Argo, Illinois.
[3] Hubbell, R. B., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutrition, *14*: 273.
[4] Primex, Procter and Gamble Company, Cincinnati.

expired CO_2 was collected in 0.5 N NaOH at one-hour intervals for either 6 or 12 hours. The NaOH was completely drained and replaced with a fresh solution in the absorption tower at each sampling. A 1-ml aliquot portion of the NaOH was mixed with 10 ml of Bray's scintillation solution[3] and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer. In some studies, samples of feces, urine, and tissues were collected for further fractionation and counting, but the results are not reported in this communication. In a few experiments, conventional protein efficiency ratios and maximal growth rates were measured. These experiments will be described under results.

RESULTS

The results of a typical experiment in which the labeled methionine was given by stomach tube are shown in figure 1. The $C^{14}O_2$ production increased to a maximum in about 2 hours and then declined to a low, very slowly decreasing rate by 6 hours. The rats receiving unheated soybeans or heated soybeans with a single dose of trypsin inhibitor had a consistently greater rate of $C^{14}O_2$ production during the first 3 or 4 hours than those receiving heated soybeans without any supplement. It had been found in earlier experiments [4] that rats receiving either unheated soybeans or heated soybeans supplemented with a single dose of trypsin inhibitor had a more rapid rate of intestinal absorption of labeled methionine than rats receiving heated soybeans alone. Therefore, it was possible that the increased rate of oxidation of the α-carbon of the methionine was merely a reflection of this increased rate of passage into the blood. To check this point, the DL-methionine-2-C^{14} was given by intraperitoneal injection. The results of this type of study are illustrated in figure 2. In essence, the same type of response was obtained as in the orally treated rats; hence it can be concluded that the altered rate of methionine oxidation was a tissue phenomenon not accounted for by rate of intestinal absorption.

A series of related studies was then carried out in which different supplements and different labeled amino acids were examined by either oral or intraperitoneal administration (table 2). Only $C^{14}O_2$ production and total C^{14} absorption calculated from intestinal contents plus fecal radioactivity are given for tissue distribution, and urinary excretion C^{14} data could not be interpreted as reflecting labeled methionine per se. A second trypsin inhibitor from soybeans, the A_1 fraction of Rackis et al. (10), gave the same response as the Kunitz-type or A_2 fraction in the Rackis classification.[5] L-Methionine-$C^{14}H_3$ gave much lower $C^{14}O_2$ recoveries, as would be expected due to the ubiquitous role of the

[3] Bray, G. A. 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem., *1*: 279.
[4] Unpublished results.
[5] This preparation was generously provided by Dr. M. L. Nesheim, Cornell University.

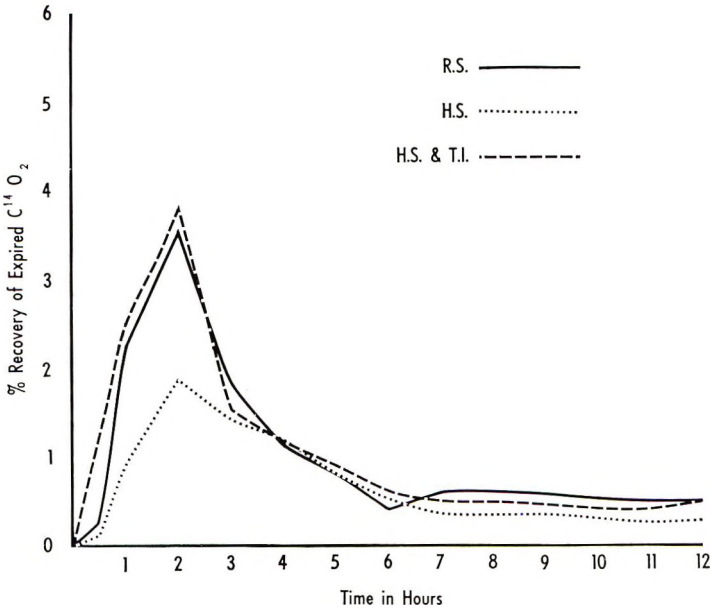

Fig. 1 Amount of $C^{14}O_2$ produced by rats following the administration of DL-methionine-2-C^{14} by stomach tube. Rats had previously been fed unheated soybeans (R.S.) or heated soybeans (H.S.). One group received a single dose of 50 mg trypsin inhibitor by stomach tube 2 hours prior to CO_2 collection.

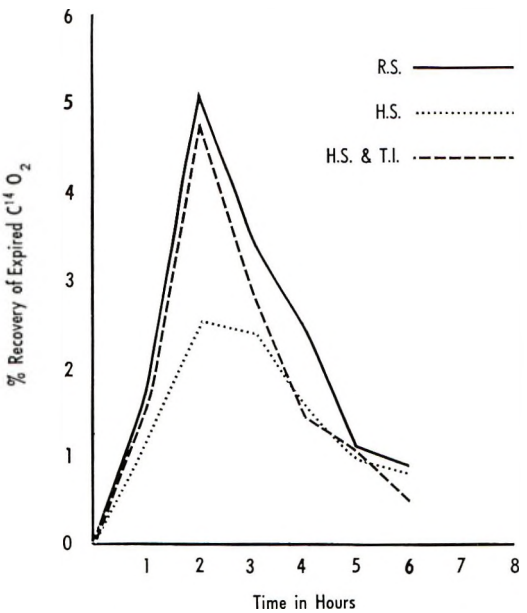

Fig. 2 Amount of $C^{14}O_2$ produced by rats following the administration of DL-methionine-2-C^{14} by intraperitoneal injection. Rats had previously been fed unheated soybeans (R.S.) or heated soybeans (H.S.). One group received a single dose of 50 mg trypsin inhibitor (T.I.) by stomach tube 2 hours prior to CO_2 collection.

methyl group in transmethylation reactions. Nevertheless, the typical increase in $C^{14}O_2$ due to trypsin inhibitor is evident. Penicillin added to the diet in an amount that consistently improves growth rate of rats receiving unheated soybeans had no effect on the stimulation of methionine oxidation caused by feeding unheated soybeans. The antibiotic effect is, therefore, not accounted for by this phenomenon. When the diet was supplemented with 0.3% DL-methionine during the 2-week feeding period, the rate of oxidation of the tracer methionine 2-C^{14} was increased, as might be anticipated as a result of adaptation. The enhanced oxidation rate due to feeding unheated soybeans is evident, but the magnitude of the effect appears to be decreased.

To determine whether the methionine oxidation alteration generally applies to other amino acids, L-lysine-2-C^{14} was used in place of DL-methionine-2-C^{14} with all other experimental conditions remaining the same. Rate of $C^{14}O_2$ was considerably lower than with labeled methionine, but the characteristic response to unheated soybeans or trypsin inhibitor was not ob-

TABLE 2

C^{14} recovery in expired CO_2 and intestinal tract plus feces following the administration of C^{14}-labeled amino acids

Exp. no.	Diet [1]	Supplement	Labeled amino acid	Recovery, % of administered activity			
				Stomach tube			Intraperitoneal
				12-hr recovery			6-hr recovery
				CO_2	Intestine + feces	Absorption	CO_2
1	H.S. [2]	—	DL-methionine-2-C^{14}	8.0	3.1	96.9	9.5
	R.S. [3]	—	DL-methionine-2-C^{14}	13.7	3.1	96.9	15.8
	H.S.	Trypsin inhibitor (A_2)	DL-methionine-2-C^{14}	13.3	3.4	96.6	12.9
2	H.S.	—	DL-methionine-2-C^{14}	8.7	3.5	96.6	
	H.S.	Trypsin inhibitor (A_1)	DL-methionine-2-C^{14}	12.0	2.9	97.1	
3	H.S.	—	L-methionine-$C^{14}H_3$				2.1
	R.S.	—	L-methionine-$C^{14}H_3$				2.9
	H.S.	Trypsin inhibitor (A_2)	L-methionine-$C^{14}H_3$				3.4
4	H.S.	—	DL-methionine-2-C^{14}	11.5	1.8	98.2	
	H.S.	Penicillin [4]	DL-methionine-2-C^{14}	10.8	2.5	97.5	
	R.S.	—	DL-methionine-2-C^{14}	14.6	2.8	97.2	
	R.S.	Penicillin	DL-methionine-2-C^{14}	13.0	3.5	96.5	
5	H.S.	DL-methionine [5]	DL-methionine-2-C^{14}	16.3	2.5	97.5	
	R.S.	—	DL-methionine-2-C^{14}	19.3	2.8	97.2	
6	H.S.	—	L-lysine-2-C^{14}	7.6	1.8	98.2	5.7
	R.S.	—	L-lysine-2-C^{14}	8.5	1.0	99.0	6.0
	H.S.	Trypsin inhibitor (A_2)	L-lysine-2-C^{14}	7.9	2.5	97.5	6.1

[1] Diet as described in table 1.
[2] Heated soybeans — steamed full-fat soy chips.
[3] Raw soybeans — uncooked full-fat soy chips.
[4] Procaine penicillin, 100 mg/100 g diet.
[5] DL-Methionine, 0.3% in diet.

served. The small differences in rate of $C^{14}O_2$ production, ranging from about 5 to 14%, were within experimental variation and far below the changes obtained with labeled methionine, which were in the order of 70%.

Oxidation of the carbon chain of methionine is known to be involved in the conversion of methionine into cysteine. One possible explanation for the increased production of $C^{14}O_2$ from the α-carbon of methionine caused by trypsin inhibitor could be an increased conversion of methionine into cysteine. To test this possibility, rats were fed the 40% heated soybean meal diet, but one-half of them received a supplement of 0.3% L-cystine in the diet. After the 2-week feeding period, followed by removal of the food for 16 hours, the rats that had received the dietary supplement were given a single dose of 50 mg L-cystine with or without trypsin inhibitor by stomach tube. All rats were given DL-methionine-2-C^{14} intraperitoneally and immediately placed in metabolism units. The $C^{14}O_2$ production data are shown in table 3. The rate of $C^{14}O_2$ production was not affected by the cystine supplement alone. However, in the absence of the cystine supplement, trypsin inhibitor increased $C^{14}O_2$ in the characteristic manner. This increase due to trypsin inhibitor was

TABLE 3

Effect of cystine supplements in the diet on C^{14} recovery in expired CO_2 following intraperitoneal injection of DL-methionine-2-C^{14}

Diet [1]	Supplement	C^{14} recovery as % of administered activity
H.S.	—	8.7
H.S.	Cystine	8.2
H.S.	Trypsin inhibitor	11.4
H.S.	Cystine + trypsin inhibitor	8.9

[1] H.S. indicates heated soybeans — steamed full-fat soy chips.

completely blocked by added cystine. The results were extremely uniform among individual rats in each group so that it can be concluded the trypsin inhibitor effect is abolished by cystine and this is interpreted as presumptive evidence that the increase in $C^{14}O_2$ from the oxidation of methionine-2-C^{14} is the resultant of an increased conversion of methionine to cysteine.

An old and repeated observation is that cystine supplements unheated soybeans so as to increase growth rate and protein efficiency. Frequently investigators studying soybean products have stated in their literature reviews that cystine supplements soybean products in general, although not to the same degree as methionine. Actually this has been a misrepresentation of the literature, for only unheated soybeans have shown an unequivocal supplementation with cystine and the usual observation has been that soybean products, well heat-treated, are unaffected by this amino acid (11, 12). This point has been checked in the present study by comparing unheated and heated soybeans at 10% protein in the diet and measuring growth rate and calculating protein efficiency ratios. These results are shown in table 4, and in confirmation of other studies, improvement was obtained only with unheated soybeans. Methionine is the first limiting amino acid in all soybean products; hence it would be expected that this amino acid would give a greater response than cystine, for the cystine content of unheated and heated soybeans is the same (table 4) and obviously the normal cystine requirement is provided by the soybean protein. If cystine is not limiting in soybean protein, and this has been shown previously to be the case with isolated soybean protein (7) as well as in the current studies, there must be an increased requirement when unheated soybeans are fed. The effect of methionine supplementation is to make up the deficiency of methionine in the protein and, in addition, provide for an increased cystine requirement in the case of the unheated soybeans. It follows that methionine should provide for a greater response than cystine alone.

Recently it has been shown that in the presence of adequate cystine, the growing rat's requirement for methionine is approximately 0.17% available methionine in the diet (7). This can be provided by feeding a diet containing 50% unheated solvent extracted soybean flakes containing approximately 25% protein. At this dietary level a plateau in growth was obtained, but the growth rate was inferior to that of rats receiving heat-treated soybeans. In these studies a further growth response could be obtained if a relatively large excess of methionine was added to the diet. If at this level of unheated soybean in the diet (50% supplying approximately 25% protein) cystine supplementation would give the same response as methionine, the methionine supplementation effect could be ruled out and the defect would then be specifically related to a cystine requirement. An experiment was designed to test this hypothesis. An unheated soybean preparation (uncooked full-fat soy chips) that had been used in other experiments was included in the semipurified diet as described in table 1, at a level that would provide 25% protein. This diet was then

TABLE 4

Cystine and methionine content of unheated and heated soybeans; growth rate and protein efficiency ratio for rats receiving diets with 10% protein for 28 days

Diet	Cystine	Methionine	No. rats	Food consumed	Gain in weight	PER [1]
	g/16 g N	*g/100 g protein*		*g/rat/day*	*g/rat/day*	
R.S.[2]	1.65	1.39	10	10.0	1.2	1.2
R.S. + 0.3% L-cystine			10	10.5	1.7	1.6
H.S.[3]	1.60	1.38	6	10.5	2.3	2.2
H.S. + 0.3% L-cystine			6	8.2	2.0	2.4

[1] PER indicates protein efficiency ratio.
[2] Raw soybeans — uncooked full-fat soy chips.
[3] Heated soybeans — steamed full-fat soy chips.

TABLE 5

Comparison of cystine and methionine supplementation of unheated soybean when sufficient soybean protein is in the diet to provide the normal methionine requirement and the effect of inorganic sulfur supplementation of unheated soybean [1]

Diet	Protein in diet	Gain in weight	Food consumed	PER [2]
	%	*g/rat/day*	*g/rat/day*	
R.S.[3]	25	4.1	—	—
R.S. + 0.3% L-cystine	25	5.1	—	—
R.S. + 0.3% DL-methionine	25	5.0	—	—
R.S.[4]	10	1.6	9.6	1.7
R.S. + 0.5% NA_2SO_4	10	1.6	9.2	1.7

[1] Four-week feeding period, 10 rats/group.
[2] PER indicates protein efficiency ratio.
[3] R.S. — unheated soybeans — uncooked full-fat soy chips.
[4] R.S. — unheated soybeans — ground dehulled soybeans.

supplemented with either 0.3% DL-methionine or 0.3% L-cystine and fed to weanling male rats for 4 weeks. The growth responses shown in table 5 were the same for the methionine and cystine supplemented groups. This experiment provides final proof that methionine utilization for growth is not impaired when unheated soybean is fed, for the cystine supplementation effect accounted for all of the growth response obtained with methionine supplementation.

Also in table 5 are results of growth and protein efficiency ratios when 10% protein from unheated soybean was supplemented with inorganic sulfate. The negative response to sulfate feeding shows that the cystine effect is not merely a reflection of an increased requirement for sulfate. Presumably the block in cystine utilization is in protein synthesis.

DISCUSSION

The metabolic defect caused by trypsin inhibitor and exhibited in an increased oxidation of methionine is undoubtedly related to an increased conversion of methioinine to cysteine. Since it has also been shown with unheated soybeans that there is a specific increase in the requirement of cystine, it follows that the metabolic defect is responsible, in part at least, for the growth depression when unheated soybeans are fed. It also follows that this is a specific effect of the trypsin inhibitors of unheated soybeans. The improvement in nutritive value by heat treatment must then be due, at least in major part, to the destruction of trypsin inhibitor and not an effect upon the remaining soybean protein. The decrease in apparent absorbability of nitrogen when unheated soybeans are fed could be accounted for by an increased metabolic fecal nitrogen due to increased intestinal secretions, although the digestion and absorption of trypsin inhibitor nitrogen per se have never been studied and could contribute.

The increased loss of nitrogen in the feces cannot, in itself account for the low nutritive value of unheated soybeans in rats. There may be a selective secretion of cystine or cystine-rich proteins into the gut with subsequent bacterial degradation of cystine as is suggested by some of the results obtained by Carroll et al. (5). In the chick, much larger nitrogen and sulfur excretion in the feces is observed when unheated soybean preparations are fed (13) and, therefore, incomplete absorption of protein may be a major contributor to low nutritive value in this species. In the rat, total sulfur excretion does not increase in the feces, but there is an increased urinary excretion of sulfur when unheated soybeans are fed (14). This has led us to the hypothesis that the increased cystine requirement is due to a metabolic block in its utilization for protein synthesis. All other possibilities of cystine utilization have not been ruled out, but it has been shown that additional dietary inorganic sulfate will not replace the need for cystine.

In 1941, Hayward and Hafner (12) presented a brilliant discussion of the prob-

able role of cystine in supplementing unheated soybeans, which appears to have been lost entirely to subsequent investigators. Basically what was accomplished in the current studies was to provide further proof for a hypothesis that they presented which postulated that a defect in cystine utilization for protein synthesis was the primary defect in unheated soybeans and that methionine supplementation acted both to provide more cystine and also to make up the specific deficiency of methionine in the protein.

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Supplementation of Cereal Proteins with Amino Acids

VI. EFFECT OF AMINO ACID SUPPLEMENTATION OF ROLLED OATS AS MEASURED BY NITROGEN RETENTION IN YOUNG CHILDREN[1,2]

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ABSTRACT As a further test of amino acid supplementation of a protein, using the amino acid levels of the FAO reference protein as a guide, children were fed diets in which rolled oats, supplemented with potentially limiting amino acids, were the sole protein source. The series began and ended with isonitrogenous, isocaloric periods in which milk served as the protein source and basal periods of the unsupplemented oat diet were interspersed between each test period. Each diet was fed for 3 successive 3-day periods. At intakes of 2 g of protein/kg, no significant effect on nitrogen retention was observed with additions to 308 mg/g N of lysine, to 296 of methionine or both together. At a level of 222 mg/g N of threonine alone or in combination, the increase was significant and the nitrogen retention only slightly below that with milk. At 1.5 g of protein/kg, some increase in retention was observed with either lysine alone or methionine alone to these levels. Threonine alone was not tested. The combination of either lysine and methionine or all 3 amino acids gave a further increase in retention. A tendency for a higher retention in a basal period following an experimental one in which methionine had been one of the supplementary amino acids was noted. This was interpreted as due to an adverse effect of methionine supplementation at the level of this amino acid in the FAO reference protein, as previously observed with the methionine supplementation of corn diets.

Since the nutritive value of a protein depends primarily upon its amino acid composition, comparison with a good amino acid pattern is a helpful way of obtaining an estimate of the deficient amino acids of most proteins. The value of such comparisons is often limited, however, because the biological availability of the amino acids must be taken into account. It is necessary, therefore, to confirm by biological trial the amino acid deficiencies of proteins for animal and human feeding. Such determinations also help to evaluate and improve amino acid reference patterns.

Using the nitrogen balance technique with young children, the effects of amino acid supplementation of lime-treated corn (1–3), and of wheat flour have been studied (4, 5). The results indicated discrepancies between the order of deficient amino acids as determined experimentally and that predicted from comparison of the amino acid pattern of the cereal protein with that of the FAO reference protein (6). For example, by comparison with the FAO reference protein, both corn and wheat are deficient in methionine. Nevertheless, children fed diets based on wheat protein showed no change in nitrogen retention when the diet was supplemented with methionine and those receiving corn consistently showed a decrease.

Because of the less marked amino acid deficiencies of rolled oats, amino acid supplementation of this protein source was chosen as a further and more sensitive test of the efficiency of the FAO amino acid reference pattern (6). Studies carried out in rats by Tang et al. (7) indicated that rolled oats are deficient in lysine, methionine and threonine, in this order, but the results of other investigators suggest that after lysine, threonine, tryp-

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tophan and methionine are next limiting to an equal extent.[4] Tang et al. (7) reported that threonine addition to rolled oats supplemented with lysine and methionine resulted in an improvement in weight gain and protein efficiency ratio (PER) because this amino acid is not completely available to the rat.

Using 6 women as subjects, Leverton and Steel (8) recently obtained nitrogen balance results using adequate protein intakes and semipurified diets whose essential amino acid patterns simulated those of the FAO reference protein and rolled oats. Their results are not comparable, however, because they used ratios of essential amino acid nitrogen to total nitrogen varying from 1:9 to 1:19 instead of the 1:4.2 specified in the FAO reference protein.

MATERIAL AND METHODS

Techniques described previously (1–5) were used to measure the effect of amino acid addition to rolled oats on nitrogen retention in children. The first of 2 series of studies involved 4 children fed 2.0 g protein/kg/day throughout ten 9-day experiments. The 3 children in the second series were fed 1.5 g protein/kg/day. The initial age, weight, protein and calorie intake of each are shown in table 1. They were fed a basal diet containing 92% of rolled oats,[5,6] 3% of glycine and 5% of cornstarch. The basal diet contained: (in per cent) 17.0 protein, 5.5 fat; 65.7 carbohydrate and 430 kcal/100 g. The added amino acids were substituted for the starch, and the nitrogen from these replaced glycine nitrogen so that all diets remained isocaloric and isonitrogenous. All of the protein was furnished by rolled oats, whereas the additional calories were provided by dextrose and margarine added to the experimental diets before cooking.

[4] Bressani, R., unpublished data.
[5] Supplied by the Quaker Oats Company, Chicago.
[6] Chemical composition of the rolled oats: (in per cent) moisture, 10.6; ether extract, 6.0; crude fiber, 2.0; nitrogen, 2.590; ash, 1.8.

TABLE 1

Age, weight, protein and caloric intake of experimental subjects

Case	Age	Weight	Protein intake	Calorie intake
		kg	*g/kg/day*	*cal/kg/day*
PC-105	4 yr 6 mo	15.14	2.0	89
PC-108	1 yr 11 mo	9.75	2.0	90
PC-110	3 yr 7 mo	12.47	2.0	89
PC-110	3 yr 3 mo	15.06	2.0	90
AT-7	1 yr 7 mo	8.05	2.0	100
AT-7	2 yr 1 mo	9.25	2.0	100
AT-7	2 yr 4 mo	10.91	2.0	100
PC-116	2 yr 1 mo	11.54	1.5	90
PC-117	2 yr 5 mo	11.17	1.5	89
PC-118	2 yr 6 mo	13.12	1.5	90

TABLE 2

Essential amino acid content of rolled oats, FAO reference pattern and rolled oats basal diet

Amino acid	Rolled oats	FAO pattern	Difference	Basal rolled oats diet
	g/g N	*g/g N*	*g/g N*	*g/g N*
Isoleucine	0.343	0.270	—	0.315
Leucine	0.515	0.306	—	0.474
Lysine	0.233	0.270	0.037	0.214
Cystine	0.135	0.270	0.049	0.203
Methionine	0.086			
Phenylalanine	0.301	0.360	—	0.410
Tyrosine	0.145			
Tryptophan	0.068	0.090	0.022	0.062
Threonine	0.203	0.180	—	0.187
Valine	0.294	0.270	—	0.270

A multivitamin and mineral capsule [7] was also given daily. The handling of the food and the collection of feces, urine and food for analysis have been described (2, 5).

The essential amino acid content in milligrams of amino acid per gram of nitrogen of rolled oats as determined microbiologically (10) and of the FAO reference protein (6) are shown in table 2. Oats contain only 82% of the methionine plus cystine, 86% of the lysine and 75% of the tryptophan contained in the FAO reference protein. Because of reports that threonine added to oat protein increased the growth of rats (7),[8] children were also fed rolled oats supplemented with threonine alone or in combination with lysine and methionine. The quantities of L-lysine·HCl, DL-methionine and DL-threonine added alone or in combination were 0.34, 0.27 and 0.20%, respectively, values which are slightly higher than needed to bring the pattern of oats to that of the FAO reference protein. In the case of threonine, 0.20% was added to meet the presumed deficiency of this amino acid due to its reduced availability (7). Amino acid proportions were the same with the 2 levels of protein intake. Tryptophan was not studied because previous results with lime-treated corn (1–3) and wheat flour (4, 5) indicated that a lower level of this amino acid than observed in oats was sufficient for maximal response.

RESULTS

Table 3 shows the effect of different amino acid additions to rolled oats as measured by the nitrogen balance method at a protein intake of approximately 2.0 g/kg of body weight/day. All nitrogen values pertaining to the same dietary treatment from all the children studied were pooled. Of the additions to the rolled oat basal diet only threonine improved nitrogen balance significantly ($P < 0.05$). When the 3 possible combinations of 2 amino acids were added to the basal diet, only those containing threonine gave higher nitrogen retention values than the basal diet. The response is not greater, however, than

[7] Gevral, donated by Lederle Laboratories, American Cyanamid Co., Pearl River, New York.
[8] See footnote 4.

TABLE 3

Effect of supplementing rolled oats with lysine, methionine and threonine alone and in combination on nitrogen balance in children fed 2.0 g of protein/kg/day

Diet	No. children tested	No. balance periods[1]	Nitrogen			N retained[2]	Significance of comparison to basal diet
			Intake	Fecal	Urine		
			mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	
Milk	4	25	327 ± 4	51 ± 3	203 ± 7	22.3 ± 1.9	$P < 0.01$
Basal	4	82	330 ± 1	68 ± 1	208 ± 3.2	16.4 ± 0.9	—
Basal + lysine	4	12	344 ± 3	72 ± 4	223 ± 8	14.1 ± 1.6	NS[3]
Basal + methionine	4	15	331 ± 3	70 ± 4	208 ± 4	15.9 ± 1.7	NS
Basal + threonine	3	10	328 ± 7	65 ± 4	193 ± 4	20.9 ± 2.0	$P < 0.05$
Basal + lysine + methionine	4	12	326 ± 4	65 ± 4	210 ± 9	15.7 ± 2.5	NS
Basal + methionine + threonine	2	6	324 ± 4	65 ± 4	199 ± 5	18.6 ± 2.4	NS
Basal + lysine + threonine	2	7	315 ± 7	61 ± 4	190 ± 12	20.7 ± 3.9	NS
Basal + lysine + methionine + threonine	2	6	328 ± 3	66 ± 2	197 ± 6	20.0 ± 2.0	NS
Milk	2	5	315	35	211	21.9	$P < 0.01$

[1] Each of 3-day duration.
[2] Average nitrogen absorbed as percentage of nitrogen intake: milk, 86.6; all basal rolled oats diet, 79.5; all basal rolled oats diet plus amino acid supplements, 79.5.
[3] NS = not significant.

TABLE 4

Effect of supplementing rolled oats with lysine, methionine and threonine on the nitrogen balance of children fed 1.5 g protein/kg/day[1]

Diet	Nitrogen			N retained[2]	Significance of comparison to basal diet
	Intake	Fecal	Urine		
	mg/kg/day	*mg/kg/day*	*mg/kg/day*	*% of intake*	
Milk	253±5	27±2	158±5	26.8±2.2	$P < 0.01$
Basal[3]	251±1	49±2	164±3	15.1±1.7	—
Basal+lysine	251±2	49±2	157±4	17.9±2.3	NS[4]
Basal+methionine	250±3	53±2	153±4	18.8±1.9	NS
Basal+lysine +methionine	244±2	51±4	129±5	26.2±2.9	$P < 0.01$
Basal+lysine +methionine+threonine	249±4	53±4	134±5	24.9±3.0	$P < 0.01$
Milk	242±2	31±3	125±8	35.5±3.6	$P < 0.01$

[1] Three children, 3 balance periods of 3-days each.
[2] Average nitrogen absorption expressed as percentage of nitrogen intake: milk, 88.1; rolled oat basal diet, 80.6; rolled oat basal diet plus amino acid supplements, 79.9.
[3] Nine balance periods of 3-days duration each.
[4] NS = not significant.

that observed from the addition of threonine alone.

No further improvement in nitrogen balance over the values observed from the threonine addition alone or from the combination of threonine with either lysine or methionine was obtained by supplementing with all 3 of these amino acids. The nitrogen balance values from threonine addition alone or in combinations were only slightly lower than those obtained from the isocaloric, isonitrogenous feeding of milk. The difference is entirely accounted for by the slightly greater nitrogen absorption with milk.

Table 4 presents the results of supplementing the rolled oat basal diet fed at a level of approximately 1.5 g/kg of body weight, a level which is considered low for the children studied. At this level there is an apparent tendency for both lysine and methionine to increase nitrogen retention when added to the basal diets although this effect fell short of statistical significance. With the combination of lysine and methionine the increase was highly significant and did not differ from that when threonine was added to the combination. The level again fell short of the average retention with milk by about the difference in nitrogen absorption between the 2 types of diets.

DISCUSSION

That the amino acid pattern of oats is better, even without supplementation, than that of corn is indicated by the higher nitrogen balances relative to milk when the oat protein basal diet was fed. The results of the present study in children indicate that instead of lysine, methionine and tryptophan as predicted from comparison with the FAO reference protein, only threonine is deficient in oats fed at a protein intake of 2.0 g/kg/day. At a level of 1.5 g protein/kg/day, both lysine and methionine also appear to be slightly deficient although only with the two added together is the difference significant.

Tang et al. (7) suggested that threonine is limiting in oats because its biological availability to the rat is only 70%. It is probable that even though acid hydrolyzates of oats appear to contain adequate amounts when judged by the 180 mg threonine/g of nitrogen in the FAO pattern, its availability to the human is sufficiently reduced that this amino acid becomes limiting in this cereal. Apparently at lower levels of nitrogen intake both methionine and lysine become limiting to some degree.

A 9-day basal period was introduced between each experimental period. This gave an opportunity to note the effect of amino acid supplementation on the nitro-

TABLE 5

Change in nitrogen retention from an experimental to a subsequent basal diet period

Basal period following:	Protein intake, g/kg/day	
	2.0	1.5
	% N intake retained	
Milk	−10.3	−16.1
Basal + lysine	− 3.5	− 1.4
Basal + methionine	+ 3.1	+ 2.0
Basal + threonine	− 3.4	—
Basal + lysine + methionine	− 0.9	—
Basal + threonine + lysine	− 5.0	—
Basal + threonine + methionine	+ 7.7	—
Basal + threonine + methionine + lysine	− 5.8	—

gen retention during the following basal period. In previous INCAP studies, the basal period tended to be lower in nitrogen retention at the same level of intake when the preceding experimental period, although isonitrogenous, furnished protein of high quality and resulted in relatively high retention. Depletion due to infection, or trauma, as well as low protein intakes, also resulted in high retentions in immediately subsequent periods even with diets of relatively poor protein quality. The poorer nitrogen retention observed by Leverton and Steel (8) in young women fed an oat diet can be explained by the overly diluted essential amino acid nitrogen to total nitrogen in the diets used by them.

In the present studies the decrease in nitrogen retention with the basal diet was greatest following either milk or the basal supplemented with threonine in any combination and least when the basal period followed methionine addition (table 5). This suggests that the added methionine may have been producing a latent imbalance which, although not sufficient to lower nitrogen retention during the experimental period of supplementation with methionine, nonetheless had an effect. This was observed in all basal periods which followed methionine addition regardless of simultaneous addition of threonine and lysine and is similar to the effect previously encountered during periods of methionine supplementation of corn. In the future attention should be given to the possible significance and value of the differences in retention of nitrogen observed on the same basal diet depending upon the quality of protein in preceding isonitrogenous periods.

Since an increased nitrogen retention during supplementation with threonine alone was evident only at low levels of protein intake, it would be pertinent to learn whether this effect is related to caloric consumption. Methionine addition to low nitrogen diets of low caloric density has been reported to decrease urinary nitrogen excretion in rats (10, 11) and dogs (12), but not in adult man (13).

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Effects of Food Restriction and Realimentation on Serum Proteins: Complement Levels and Electrophoretic Patterns[1]

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ABSTRACT The effects of food restriction and refeeding on the hemolytic complement activity and electrophoretic patterns of the serum proteins of adult, male, Sprague-Dawley rats were investigated. Groups of rats, fed 50% of the food required for weight maintenance, were bled following weight losses of 12, 26 and 33%. Other groups were repleted following depletion and bled when their initial weight was regained. Complement levels became significantly decreased in the 12% depleted group, but increased in the 26 and 33% groups. Values for the repleted groups were in the normal range. The depletion regimen caused significant decreases in total serum protein, albumin, α_1- and β-globulins while α_2- and γ-globulin levels were either normal or increased. Total protein, albumin and α_1-globulin concentrations remained significantly low in the repleted groups whereas α_2- and γ-globulins were either normal or significantly increased. It was concluded that (a) in semi-starvation states there is a conservation of serum components which are involved in native and acquired resistance to infectious agents; and (b) albumin, α_1- and β-globulins provide a labile reserve of proteins for tissue utilization and maintenance.

Increased interest in serum complement is shown by the recent publication of several reviews on the subject (1–5). The role of complement in both native and acquired resistance to infectious agents is well established (3, 4). Notwithstanding the importance of good nutrition in resistance to disease (6, 7), the effects of various dietary regimens on complement levels have received little attention. Studies on the influence of diet have been primarily concerned with the effects of ascorbic acid on complement levels in guinea pigs (3, 4).

Although rat serum contains hemolytic complement of relatively high activity (8), most workers have used the rat only in studies concerned with species comparisons and the interchangeability of complement components (4, 8). Inasmuch as the rat is a more suitable test animal for many nutritional studies, this species has been used in the current study in which the effects of food restriction and refeeding on serum complement levels and electrophoretic patterns were investigated.

EXPERIMENTAL

Adult, male rats of the Sprague-Dawley strain were used. The animals were caged singly in suspended wire cages with wire bottoms and were maintained with a diet of commercial laboratory chow[2] and tap water. Two days prior to the initiation of the experiment a baseline blood sample of 5.0 ml was obtained from approximately 20% of the animals (group A1) in each of the groups. The samples were collected from lightly anesthetized rats by cardiac puncture.

One group (A2) of rats was fed 22 g/day of the stock diet, an amount adequate for weight maintenance. The other groups (B, C, D) were fed one-half this amount until weight losses of 12, 26, 33%, respectively, were obtained. At this time one-half of the rats were bled and the remainder were fed the stock diet ad libitum. Blood samples were also obtained upon weight repletion.

Hemolytic complement activity was determined by a modification (9) of the 50% end-point method of Kent et al. (10). Protein, electrophoretic and hematologic anal-

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

TABLE 1

Summary of weight data on adult rats relative to food restriction and refeeding

	Group	No. rats	Original wt	Depletion wt	Depletion time	Final wt	Repletion time
			g	*g*	*days*	*g*	*days*
A1	Baseline bleeding (normal control)	22				406 ± 4.9	
A2	Age and wt control	14	410 ± 7.7[1]		(34)[2]	409 ± 8.1	
B1	12% Depleted	15	409 ± 5.5	356 ± 4.7[3]	6		
B2	12% Depleted:repleted	15	406 ± 4.8	358 ± 5.4[3]	6	405 ± 5.3	4
C1	26% Depleted	14	405 ± 6.7	298 ± 3.8[3]	22		
C2	26% Depleted:repleted	14	400 ± 4.0	298 ± 3.3[3]	22	401 ± 4.1	6
D1	33% Depleted	13	412 ± 8.3	275 ± 4.4[3]	27		
D2	33% Depleted:repleted	13	411 ± 7.2	275 ± 4.6[3]	27	399 ± 5.8	10

[1] Values include SE.
[2] Number of days animals were fed the restricted diet.
[3] Statistically significant difference from baseline values (group A1) is indicated: $P < 0.01$.

TABLE 2

Summary of hematologic and serum complement data on adult rats relative to different regimens of food restriction and refeeding

	Group	Hemoglobin	Hematocrit	Serum complement
		g/100 ml	%	$C'H_{50}$
A1	Baseline bleeding	13.5 ± 0.16[1]	45 ± 0.5	46 ± 2.0
A2	Age and wt control	14.0 ± 0.25	45 ± 0.9	43 ± 1.1
B1	12% Depleted	14.7 ± 0.18[2]	50 ± 0.8[2]	38 ± 1.3[3]
B2	12% Depleted:repleted	13.1 ± 0.19	36 ± 0.9[2]	46 ± 2.4
C1	26% Depleted	14.2 ± 0.30[3]	50 ± 1.0[2]	58 ± 2.0[2]
C2	26% Depleted:repleted	11.4 ± 0.14[2]	39 ± 0.5[2]	47 ± 2.4
D1	33% Depleted	15.1 ± 0.20[2]	49 ± 0.7[2]	53 ± 1.8[3]
D2	33% Depleted:repleted	11.5 ± 0.18[2]	41 ± 0.9[2]	47 ± 3.1

[1] Values include SE.
[2] Statistically significant difference from baseline values (group A1): $P < 0.01$.
[3] Statistically significant difference from baseline values (group A1): $P < 0.05 > 0.01$.

yses were carried out by procedures described previously (11). Analysis of the data was made by standard statistical procedures, using the *t* test (12).

RESULTS AND DISCUSSION

General and weight data are summarized in table 1. Feeding the restricted diet caused a rapid weight loss, 9 g/day, during the early stage of depletion (B1). The rate of loss became stabilized at a lower level, 5 g/day, as the rats adjusted to the dietary regimen (C1, D1). The introduction of the ad libitum feeding induced prompt gains in body weight. The rate of gain of the 26% depleted-repleted group (C2) was appreciably greater than that of the comparable 12% (B2) or 33% (D2) groups.

The results of hematologic and serum complement analyses are presented in table 2. Food restriction caused a marked and uniform hemoconcentration in all depleted groups which was not proportional to the amount of weight lost. Refeeding was accompanied by a conspicuous hemodilution which was inversely related in general to the time required for weight repletion. Similarly, hemoglobin values increased significantly in all depleted groups and decreased in repleted groups with the exception of group B2.

Figs. 1 a, b Effects of food restriction (solid line) and refeeding (dashed line) on serum protein concentrations.

Key: ○ = total serum protein; ◒ = α_2-globulin; ■ = albumin; ● = β-globulin; ◓ = α_1-globulin; □ = γ-globulin

Statistically significant differences from baseline values (group A1) are indicated: superscript 1, $P < 0.01$; superscript 2, $P < 0.05 > 0.01$.

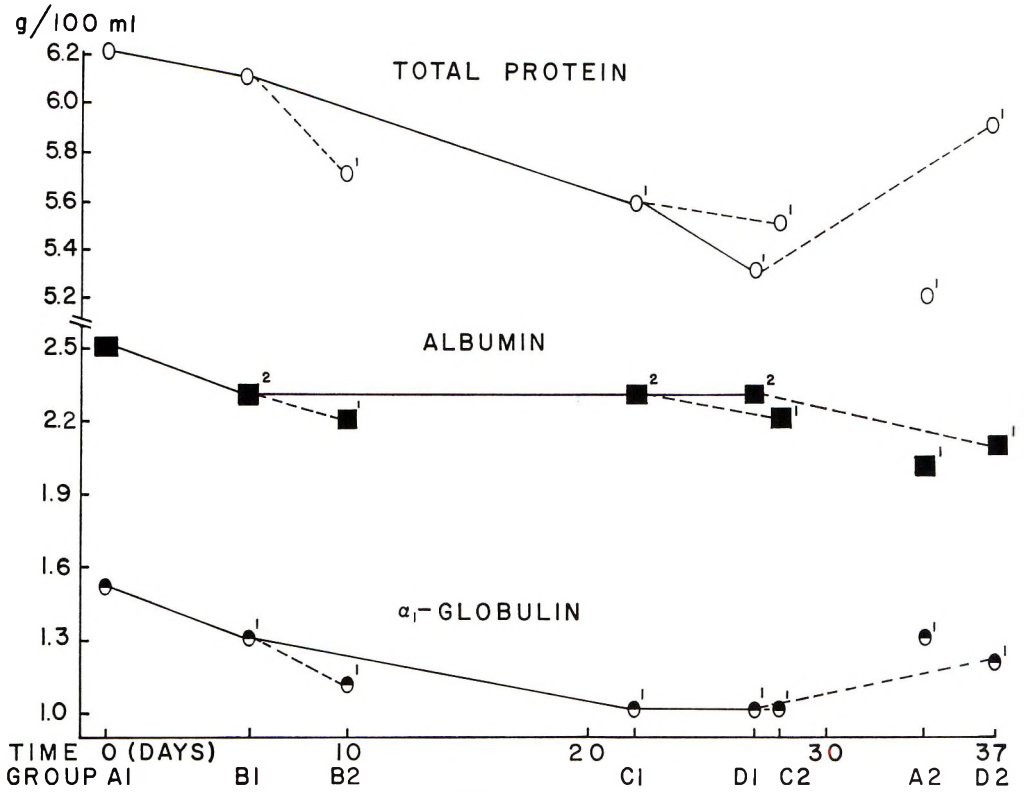

Figure 1a

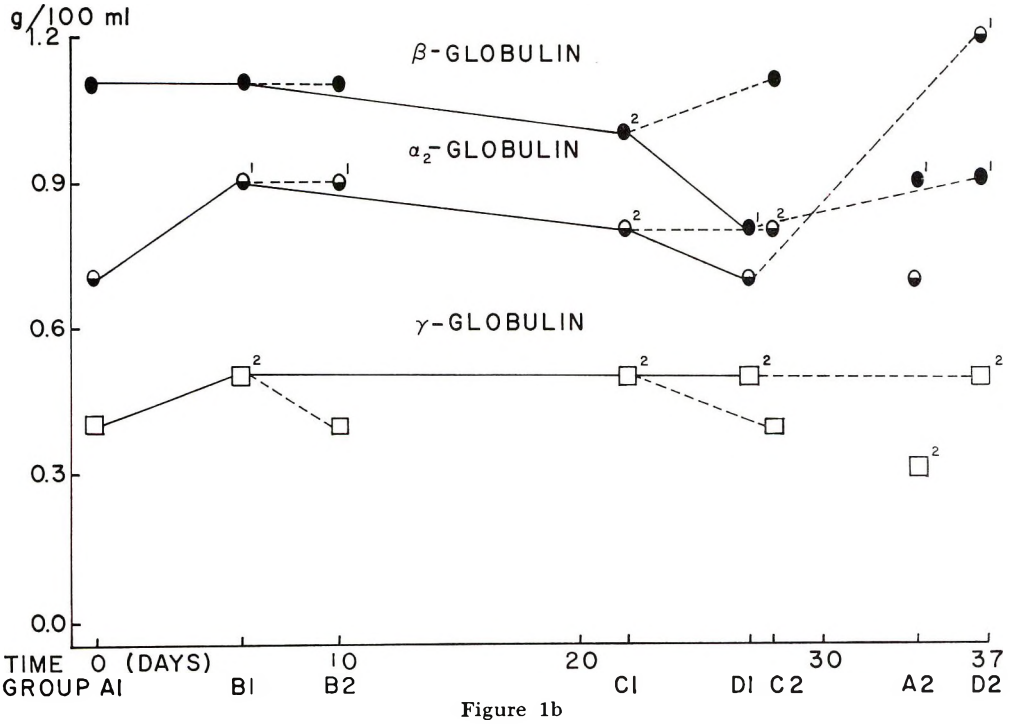

Figure 1b

Depletion at the 12% level (B1) caused a significant reduction in hemolytic complement activity, although further depletion elicited pronounced increases in serum complement concentrations. The complement values for the 26% (C1) and 33% (D1) groups were not only significantly greater than those of group B1 but also of the control groups A1 and A2. The increase in the hemolytic complement activity of serum during moderate (C1) and severe (D1) depletion suggested that there might be either a release of complement from tissues or a decrease in a serum inhibitor under these conditions. Restoration of body weight was accompanied by a uniform return of complement activity to normal in all repleted groups irrespective of the depletion level.

The effects of the dietary regimens on the partition of serum proteins are shown in figures 1a and b. Total serum protein concentrations were not altered following a 12% weight loss (B1). Significant changes, however, occurred in the albumin, α_1-, α_2-, and γ-globulin fractions which tended to offset each other. Further depletion (C1, D1) was characterized by significant reductions in total serum protein levels referable to dimunition of albumin, α_1- and β-globulin concentrations.

Weight restoration was accompanied by variable patterns of response of the serum protein fractions in the repleted groups. The responses were influenced by the extent of prior depletion and by the degree of hemodilution. Total serum protein concentrations either declined (B2) or increased significantly (D2) or remained essentially the same (C2). The conclusion that the observed changes were not primarily a result of varying degrees of hemodilution was supported by the divergent responses of the subfractions of serum following realimentation.

In group B2, repletion was characterized by further reductions in the concentration of the albumin, α_1- and γ-globulin fractions, whereas the α_2- and β-globulins remained at depletion levels. In group C2, the albumin and γ-globulin declined, the β-globulin increased and the α_1- and α_2-globulin concentrations remained unchanged. In the most drastically depleted group, D2, the increase in the concentration of the α_2-globulin fraction was most pronounced, although the elevation of the α_1-globulin was also highly significant. Albumin was the only fraction to undergo a conspicuous decline.

The pronounced serum protein alterations observed in the age and weight control group (A2) were unexpected in view of the normal values for hematocrit, hemoglobin and complement. All serum fractions with the exception of the α_2-globulins were significantly reduced notwithstanding the lack of change in body weight. It may be inferred from these observations that: (a) tissue maintenance has a higher priority in the organism's metabolic processes than the serum proteins; (b) a diet adequate for weight maintenance is inadequate for maintenance of several serum protein fractions; and (c) the albumin, α_1- and β-globulins of serum provide a labile reserve of proteins for tissue utilization. The observation of a normal complement level (A2) may reflect a conservation of factors involved in native and acquired resistance to infectious agents.

The results of the current study confirm and extend an earlier observation of Rice and Annau (13) with respect to the lack of correlation between hemolytic complement activity and the concentrations of serum protein fractions. Working with guinea pigs, they noted no correspondence between complement levels and the concentration of albumin or globulin fractions separated by salt precipitation. Using a more discriminating method of separation, we observed no parallelism between complement levels and the major fractions of serum. The lack of correlation may be attributed to the fact that the proteins constituting the complement complex represent such a small percentage of the total serum proteins ($< 1\%$) that the electrophoretic method used would not detect them.

A comparison of the results of the electrophoretic analyses for the 26% depleted-repleted group (C2) with the corresponding values of repleted rats previously subjected to the same loss in weight by feeding a protein-free diet (11) demonstrated the marked influence of previous dietary history. In the latter group (11), total protein, albumin, α_1-, α_2- and β-glob-

ulin concentrations were restored to their normal range upon weight repletion; however, in group C2 total protein, albumin and the α_1-globulin levels remained significantly depressed. It may be inferred that semi-starvation has a greater debilitating effect upon the protein synthetic mechanisms of the organism than a simple protein deficit.

A longitudinal study allows an estimate to be made of the relative sensitivity and resistance of the various serum fractions to nutritional influence. Although quantitatively the largest of the serum fractions, albumin was less affected by the depletion regimen than the α_1- or β-globulins. The α_1-fraction declined 33% in the moderate (C1) and severely (D1) depleted groups and the β-globulins had decreased 26% in group D1. The α_2- and γ-globulins were not only resistant to the depleting effects of the restricted diet but increased in concentration, an observation that may be attributed in part to the concomitant hemoconcentration. Upon repletion, increased levels of the latter 2 fractions were observed for the 33% depleted group (D2), whereas the other serum components remained significantly low.

The results of the present study are at variance with the observations of Pruzansky and Axelrod (14) and of Wertman et al. (15) with respect to the effects of partial inanition on serum complement levels. Both groups of workers (14, 15) observed decreased hemolytic complement activity in their inanition control groups, whereas significantly increased complement titers were observed in the current investigation in moderately (26%) and severely (33%) depleted rats.

Several factors may be cited that might explain the divergent results: (a) the above workers (14, 15) used weanling rats to initiate their experiments, whereas we used large, young, adult males (it might be anticipated that the ability of the adult rat to resist and to recover from nutritional stress would be much greater than that of the weanling); (b) it is also proposed that complement stores in tissues would be at a higher level in the adult than in the young animal; (c) in both of the cited studies (14, 15), serial determinations were not made (no data are available for comparisons at intermediate points in the depletion regimens); (d) in the former study (14), the inanition controls maintained a constant weight, whereas in the latter (15) their weight doubled. The maintenance of a constant weight (cf. group A2) had no effect on complement titers in adult rats.

The studies reported above suggest that there is a conservation of serum constituents (complement, γ-globulin) which are involved in native and acquired resistance to infectious agents. The physiologic functions of the α_2-globulins are still obscure, but their maintenance under conditions of severe nutritional stress can imply an important role in the organism's survival. The experimental conditions investigated reflect a nutritional situation observed in many underdeveloped countries and in war-time and post-war periods.

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Adenosine Triphosphatase and ATP-P_i Exchange in Mitochondria of Essential Fatty Acid-Deficient Rats[1,2]

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ABSTRACT ATPase, Mg^{++}-ATPase, DNP-ATPase, and ATP-P_i exchange was measured in liver mitochondria from normal and essential fatty acid-deficient rats. Little difference was noted in these systems when freshly prepared mitochondria were used. When the mitochondria were aged at 30°C for various times, however, differences between the normal and deficient mitochondria became apparent. Uncoupling of oxidative phosphorylation occurred relatively rapidly in deficient mitochondria that were aged, and this appeared to be linked to a loss of ATP-P_i exchange activity, but not to ATPase activity.

In earlier studies it was observed that liver mitochondria from essential fatty acid-deficient rats (deficient mitochondria) exhibited an uncoupled oxidative phosphorylation. They swelled much more rapidly on aging at 30° than did normal ones, and appeared to be swollen when obtained by the usual methods (1–3). Hayashida and Portman (4) have made further observations on the swelling characteristics of deficient mitochondria, and others have demonstrated an increased metabolic rate (5), cytochrome oxidase (6), and mitochondrial dehydrogenase activity (7) associated with essential fatty acid deficiency (EFA) in rats.

In the present study, liver mitochondria from EFA-deficient rats were compared with normal mitochondria, with respect to ATPase, and ATP-P_i exchange. A functional relationship between these enzyme systems and oxidative phosphorylation has been indicated by work in a number of laboratories (8, 9).

METHODS

An essential unsaturated fatty acid deficiency was produced in rats, and mitochondria were prepared as described elsewhere (10, 11), taking increased precautionary measures to conclude the preparative procedures rapidly, and to keep all materials as cold as possible. They were suspended in cold 0.25 M sucrose and used as enzyme immediately, or were aged by keeping them in 0.25 M sucrose at 30°C for varying periods of time. ATPase was assayed by measuring the release of P_i from ATP in the presence or absence of 2-4,dinitrophenol (DNP) or Mg^{++}, and ATP-P_i exchange was measured as described by Cooper and Lehninger (12). Oxidative phosphorylation was measured as described previously (1).

RESULTS

No differences were observed between freshly prepared normal and deficient mitochondria in ATPase, Mg^{++} activated ATPase, DNP activated ATPase, or ATP-P_i exchange (table 1). The activities of these enzyme systems changed considerably in the mitochondrial preparations that were aged at 30°C for 30 minutes, and aging affected the deficient mitochondria differently than it did the normal ones. For example, the DNP-ATPase activity of aged deficient mitochondria was significantly ($P < 0.01$) less than that of aged normal mitochondria. ATP-P_i exchange activity in both mitochondria preparations was sharply reduced as a result of the aging, but in normal mitochondria it was significantly greater ($P < 0.05$) than that observed in the deficient mitochondria. Measurements of the extremely low levels of ATP-P_i exchange observed in the aged

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TABLE 1

ATPase and ATP-P_i exchange activities in liver mitochondria from normal and fat-deficient rats[1,2]

	Freshly prepared mitochondria		Aged mitochondria	
	Normal	Deficient	Normal	Deficient
ATPase	688 ± 213[3]	757 ± 242	1868 ± 316	1324 ± 801
ATPase + Mg^{++}	919 ± 213	1192 ± 242	3460 ± 386	4451 ± 801
ATPase + DNP	4112 ± 340	4134 ± 423	2212 ± 187	1054 ± 270
ATP-P_i exchange	749 ± 95	729 ± 156	10 ± 2.5	2 ± 0.4
ATP-P_i exchange + Mg^{++}	976 ± 183	1018 ± 153	166 ± 66	25 ± 13
ATP-P_i exchange + DNP	58 ± 6.0	33 ± 6.7	13 ± 5.7	1.6 ± 0.5

[1] Nine animals/group. ATPase values are millimicromoles of P_i released. ATP-P_i exchange values are millimicromoles of ATP^{32} formed.

[2] The following were present in the ATPase incubation medium: ATP, 0.01 M; Mg^{++} (when used), 0.003 M; DNP (when used), 0.0004 M; histidine buffer, pH 7.0, 0.03 M; mitochondria from 50 mg tissue, 1.0 ml final volume. Incubation was carried out for 20 minutes at 23°C. P_i was determined according to the method of Martin and Doty (13), modified to use one-fifth the volume of each ingredient they described.

[3] Mean ± SE of mean.

preparations were subject to error, however, and the reliability of the absolute values shown may be questioned.

In an attempt to determine whether the changes with aging of the enzyme systems under consideration occurred concurrently with a loss of phosphorylation capacity in the 2 preparations, the experiment shown in table 2 was performed. A gradual decrease in oxidative phosphorylation was observed in normal mitochondria that were aged at 30°C for increasing times, whereas an almost precipitous loss in oxidative phosphorylation capacity occurred in the deficient mitochondria similarly treated. There appeared to be no abrupt changes in either ATPase or Mg^{++}-ATPase that obviously might account for the precipitous reduction in oxidative phosphorylation.

A sharp reduction in ATP-P_i exchange occurred in both mitochondria preparations after aging for 15 minutes. The deficient mitochondria differed, however, in that the exchange activity at the end of this period was almost imperceptible, whereas the exchange activity in the normal mitochondria after a similar aging was approximately one-third that observed in fresh preparations.

DISCUSSION

In work subsequent to that reported earlier (1), we observed that if extreme care was exerted, it was possible to prepare liver mitochondria from EFA-deficient rats that demonstrated less uncoupling than previously described. Our experience in this respect has been similar to that of Weinbach (14), who prepared mitochondria that exhibited increased oxidative phosphorylation stability, by exerting greater care in their isolation. The extreme lability of oxidative phosphorylation in liver mitochondria from rats rendered deficient in unsaturated fatty acids is demonstrated in the experiments reported here. Although the shortest aging period shown in table 2 is 15 minutes, we have observed preparations of deficient mitochondria in which there was almost no oxidative phosphorylation capacity after only 7.5 minutes' aging at 30°C.

The present experiments did not reveal any unique relationship between oxidative phosphorylation and ATPase activity in deficient mitochondria that was not noted in normal mitochondria. The differences observed in ATP-P_i exchange capacity in the 2 preparations, upon aging, (table 2) may be of significance, however, and worthy of comment. Although there was a rapid decrease in ATP-P_i exchange activity in normal mitochondria aged at 30°C, there was not a corresponding loss of oxidative phosphorylation capacity. Weinbach (14) observed a similar relatively rapid decline in the exchange reaction as compared with oxidative phosphorylation in liver mitochondria. Apparently, there is a minimal exchange capacity essential for oxidative phosphorylation, which is lost by the deficient mitochondria upon relatively short periods of aging at 30°C. A further demonstration of the lability of the ATP-P_i exchange reaction in deficient mitochondria was observed in experiments in which different portions of mitochondrial preparations were aged for 30 minutes at various temperatures. The stability of the exchange reaction decreased with increasing

TABLE 2

Changes with aging in mitochondrial enzyme activities[1,2]

Aging times in minutes	Normal liver					Deficient liver				
	0	15	30	60	90	0	15	30	60	90
Oxidative phosphorylation										
Succinate	1.63	1.39	1.35	1.09	0.8	1.38	0.05	0.10	0	0
β-Hydroxybutyrate	2.35	1.91	2.06	1.80	1.80	1.22	0	0	—	—
α-Ketoglutarate	2.59	2.14	1.03	1.25	0.5	1.72	0	0	—	—
ATPase	678	2070	1650	1230	876	707	1869	1162	780	670
ATPase + Mg^{++}	1130	2480	4730	5130	4550	1700	4700	5420	5500	5600
ATP-P_i exchange	203	64	40	34	21	208	8	4	2	—

[1] Oxidative phosphorylation data are P/O ratios. See footnotes to table 1 for ATPase and ATP-P_i exchange units. The data show changes with aging which are typical of those observed in 6 separate experiments.

[2] Mitochondria were prepared as described (see text), resuspended in 0.25 M sucrose, and the suspensions rapidly brought to 30°C. Portions were removed at various time intervals, for the determinations indicated. Oxidative phosphorylation was determined manometrically (1). Substrates were used at a level of 21 μmoles/flask. ATPase and exchange activities were determined as described in table 1.

temperature of incubation; a loss of approximately 50% in exchange activity occurred at a temperature of about 17°C in the deficient mitochondria, whereas a similar loss in normal mitochondria did not take place until the incubation temperature was 22 to 23°C.[3]

The relationship of the ATP-P_i exchange reaction to oxidative phosphorylation has been considered in some detail (8, 9). Although the hypotheses set forth to account for respiratory chain phosphorylations differ from one another in their concept of the roles of intermediates involved in bond energy transfers, the presumed site or sites of action of DNP are such that in any case it might be expected to affect the exchange reaction. Löw et al. (15), however, observed that under certain conditions, treatment of mitochondria with DNP resulted in a marked depression of the exchange reaction without concomitant loss of phosphorylation. The question arose, from these considerations, whether the differences in phorphorylation and exchange activities (table 2) between fat-deficient and normal mitochondria might be explored further by a study of the action of DNP on ATP-P_i exchange in the 2 preparations. We used DNP in concentrations up to 30 μM, but were unable to detect any difference between normal and deficient mitochondria in its effect on the exchange reaction. There was a marked difference in the effect of DNP in low concentrations on respiratory control in the two preparations, however.[4]

Considering the ease with which deficient mitochondria swell with aging, it is tempting, in view of the interrelation between structural changes in mitochondria and electron transport and oxidative phosphorylation, to conjecture that an essential unsaturated fatty acid deficiency results in a structurally defective mitochondrion. This manifests itself, perhaps among other ways, in a more easily disrupted ATP-P_i exchange. Experiments are in progress to investigate this and related phenomena further.

[3] Ito, T., and R. M. Johnson, unpublished experiments.

[4] See footnote 3.

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Interrelationships of Ascorbic Acid and Pantothenic Acid in the Young Guinea Pig[1]

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ABSTRACT Two- to four-day-old male guinea pigs, weighing from 68 to 112 g, were fed a semi-synthetic ration along with 4 levels of ascorbic acid: 0, 2, 10, and 40 mg/day and 4 levels of calcium pantothenate: 0, 0.06, 0.2 and 1 or 8 mg/day to observe whether an interrelationship existed between the 2 vitamins in the guinea pig. Ascorbic acid in blood serum and adrenal glands reflected dietary intake of this vitamin. Hemoglobin and packed red blood cell volume increased slightly with a dietary increase of each vitamin. Pantothenic acid in the urine reflected intake of this vitamin. When calcium pantothenate was included in the diet, blood and liver values were higher than when it was omitted. Blood pyruvic acid was not correlated with concentration of pantothenic acid in blood or diet. Acute pantothenic acid deficiency symptoms included soft wooly fur, lethargy, diarrhea, convulsions and hemorrhagic adrenals. Large amounts of calcium pantothenate did not prevent symptoms of scurvy, and large amounts of ascorbic acid were not beneficial in pantothenic acid deficiency; neither did large amounts of either of the 2 vitamins have any effect on weight gain, food intake or efficiency of food utilization when compared with the smallest amounts given in these experiments.

In 1951 Daft[3] reported that when weanling rats were fed a pantothenic acid-deficient diet which was supplemented with 2% ascorbic acid, they showed either no pantothenic acid deficiency symptoms, or the development of the deficiency syndrome was "at the least greatly delayed or modified." Later Hundley and Ing[4] found that this effect was not specific for ascorbic acid. Normally the rat is able to synthesize as much ascorbic acid as it needs, but requires a dietary source of pantothenic acid whereas the guinea pig requires a dietary source of both ascorbic acid and pantothenic acid.

Since the development of a satisfactory purified diet for guinea pigs (1), it has become possible to study the members of the vitamin B complex and their interrelations with other nutrients in the nutrition of the guinea pig. In 1954 Reid and Briggs (2) reported that "the sparing effect of large amounts of dietary ascorbic acid on pantothenic acid deficiency in the rat could not be duplicated in the guinea pig." These authors conceded that "Further studies are required, however, before drawing the conclusion that in diets containing sub-optimum amounts of pantothenic acid no benefit is derived from the presence of dietary ascorbic acid in excess of the amount necessary to prevent scurvy." Since Reid and Briggs used growth and survival as their main criteria, it seemed worthwhile to reinvestigate the possibility that dietary ascorbic acid could have a sparing effect on the pantothenic acid-deficient guinea pig. The possibility of a sparing effect of pantothenic acid on the scorbutic guinea pig was investigated also.

METHODS

Twenty-one male guinea pigs, 2 to 4 days old and weighing 68 to 116 g, were obtained from a commercial source. The experimental design was of the Latin square type with 2 vitamins as the variables and was replicated twice. Amounts of the vitamins in mg/day were ascorbic acid: 0, 2 and 40; calcium pantothenate: 0, 0.06 and 1 or 8. Instead of the maximal amounts of the 2 vitamins, control animals received 10 mg ascorbic acid and

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[3] Daft, F. S. 1951 Effect of vitamin C on pantothenic acid-deficient rats. Federation Proc., *10:* 380 (abstract).

[4] Hundley, J. M., and R. B. Ing 1953 Prevention of pantothenic acid deficiency with glucuronolactone and other compounds related to ascorbic acid. Federation Proc., *12:* 417 (abstract).

0.2 mg calcium pantothenate. The experiments lasted 30 and 33 days. One animal in the first experiment, receiving daily supplements of 2 mg ascorbic acid and 0.06 mg calcium pantothenate, died of a lung infection; therefore no data were obtained for this pig.

The purified ration was a modification of that used by Reid and Briggs (1) and was composed of the following: (in %) casein (vitamin-free), 30; glucose, 11.6; dextrin, 11.7; gum arabic, 15; corn oil, 7.3; salts,[5] 5; potassium acetate, 2.3; magnesium oxide, 0.5; inositol, 0.2; choline chloride, 0.2; and vitamins.[6] The ingredients were mixed together and stored at −20°C. As needed, 400-g lots were mixed with 35 to 40 ml of distilled water, rolled out, and cut into bite-size pieces. The ration was spread out on trays and dried, with frequent turning, in an air oven for approximately 3 hours at a temperature below 55°C. When thoroughly dried, it was cooled and stored in tightly covered containers in a freezer. Approximately 0.25 μg of vitamin B_{12} plus the varying amounts of calcium pantothenate and ascorbic acid were fed orally each day. Ascorbic acid was dissolved in 70% glucose, calcium pantothenate in distilled water. When 40 mg of ascorbic acid were fed, it was divided into 4 doses, whereas 0.2 mg of calcium pantothenate was fed in 2 doses, 8 mg of calcium pantothenate (in the first experiment) in 4 doses, and 1 mg of calcium pantothenate (in the second experiment) in 5 doses. All other amounts of the 2 vitamins were given in single doses.

The animals were weighed daily and food consumption was recorded. At weekly intervals blood samples were taken from an ear vein for blood plasma ascorbic acid determinations. Each week urine was collected for 48 hours and analyzed for pantothenic acid. At the conclusion of each study the following determinations were made: packed red blood cell volume, hemoglobin, serum ascorbic acid, blood pyruvic acid, ascorbic acid in the adrenal glands and pantothenic acid in the blood and liver. The appearance of each animal was noted at autopsy. Scorbutic animals were killed when symptoms of scurvy were so pronounced that death appeared to be imminent. The animals were anesthetized with an intraperitoneal injection of a solution of sodium pentobarbital. Blood samples were obtained from the portal vein. Methods used for blood and tissue analyses were described in a previous paper (4).

RESULTS AND DISCUSSION

Guinea pigs fed neither ascorbic acid nor calcium pantothenate developed scorbutic symptoms within 18 days, whereas those fed calcium pantothenate but not ascorbic acid developed symptoms within 23 days. Hence it appears that when no ascorbic acid was fed, calcium pantothenate delayed the appearance of scorbutic symptoms 5 days. Large amounts of calcium pantothenate did not prevent symptoms of scurvy, however.

The largest amount of ascorbic acid (40 mg/day) did not protect the animals getting no calcium pantothenate against pantothenic acid deficiency. Symptoms of pantothenic acid deficiency included lethargy, soft wooly fur, diarrhea, convulsions and hemorrhagic adrenals.

Control animals, fed 10 mg of ascorbic acid and 0.2 mg of calcium pantothenate each day, gained weight at an everage of 4.8 g/day during the course of the experiment. Their weight gains compared favorably with those of animals of similar initial weights, fed a similar diet, maintained by Reid and Briggs (2).

No trends in weight gain, food consumption or food efficiency were observed with increasing amounts of either of the 2 vitamins. Comparisons made between pigs getting neither ascorbic acid nor calcium pantothenate and those getting large amounts of ascorbic acid but no calcium pantothenate indicated that deficiency symptoms appeared in both groups of pigs within 18 days. Reid and Briggs (2) reported that guinea pigs subjected to a lack of both ascorbic acid and pantothenic acid in the diet, died earlier than guinea pigs with either deficiency alone.

Hemoglobin and packed red blood cell volume increased slightly with intake of

[5] Phillips and Hart (3).

[6] Vitamins were added in amounts to provide the following in mg/kg of diet: thiamine·HCl, 16; riboflavin, 16; pyridoxine·HCl, 16; nicotinic acid, 200; biotin, 0.6; folic acid, 10; α-tocopheryl acetate, 20; 2-methyl-naphthoquinone, 2; vitamin A 18,000 USP units; and vitamin D, 2,550 USP units.

each of the 2 vitamins. Pigs getting no ascorbic acid had the lowest average hemoglobin value, 10.85 g, whereas those getting 10 and 40 mg averaged 11.94 g/100 ml of blood. Animals fed no calcium pantothenate averaged 11.28 g, and those fed 0.2 and 1 or 8 mg averaged 11.82 g/100 ml of blood. Data for packed red blood cell volume followed the same trends, and average values ranged from 32.6 to 36.3%. Anemia was characteristic of animals after they had been fed the antimetabolite, ω-methylpantothenic acid, for 47 days in a previous study (4) but was not observed in animals whose diets were lacking in pantothenic acid in this study. This may indicate that anemia can be produced only when pantothenic acid deficiency is developed over a longer period of time.

Serum ascorbic acid (table 1) was related to intake of the vitamin as was the concentration of ascorbic acid in the adrenal glands. Increasing the amount of dietary calcium pantothenate did not affect the concentration of ascorbic acid in either blood serum or adrenal glands.

The concentration of pantothenic acid in the blood ranged from 13 to 50 μg/100 ml; in the liver, from 78.7 to 143.9 μg/g and in the urine from 3.0 to 283.2 μg/day (table 2). In general, when calcium panto-

TABLE 1

Ascorbic acid concentrations of blood serum and the adrenal glands

Supplements		Ascorbic acid values[1]					
		Blood serum					
		Week of study					Adrenal glands
Ascorbic acid	Calcium pantothenate	1	2	3	4	5	
mg/day	*mg/day*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*	*mg/100 g*
0	0	—	0.09	0.05	—	—	13.78
0	0.06	0.26	—	0.06	—	—	9.84
0	1 or 8	—	0.06	0.08	0.10	—	10.34
2	0	—	0.10	0.19	0.10	0.09	31.31
2	0.06	—	0.12	0.15	0.24	0.08	36.05
2	1 or 8	—	0.14	0.25	0.16	0.15	47.21
10	0.2	0.41	0.28	0.19	0.26	0.20	80.55
40	0	—	0.42	0.69	—	—	67.65
40	0.06	0.29	0.41	0.71	0.51	0.43	119.42

[1] Individual values represent from 1 to 3 animals.

TABLE 2

Concentration of pantothenic acid in blood, liver and urine

Supplements		No. of animals	Pantothenic acid		
Ca pantothenate	Ascorbic acid		Blood	Liver	Urine[1]
mg/day	*mg/day*		*μg/100 ml*	*μg/g*	*μg/day*
0	0	3	24[2]	97.0	9.4[2]
0	2	3	16	96.6	7.6[2]
0	40	3	25[3]	114.8	5.7[2]
0.06	0	2	48[3]	115.3	14.0
0.06	2	1	20	91.5	7.2
0.06	40	2	26[3]	143.9	19.2
0.20	10	2	32	102.4	14.4
1	0	1	—	140.0	31.4
1	2	1	39	122.7	29.0
8	0	1	50	124.6	199.6
8	2	1	32	78.7	114.5

[1] Mean values of weekly 48-hour collections.
[2] Figure is mean for 2 animals.
[3] Figure represents one animal.

thenate was included in the diet, blood and liver values were higher than when it was not fed; however, there were wide variations in values. The concentration of pantothenic acid in the urine reflected the intake of this vitamin, but animals fed no calcium pantothenate excreted fairly large amounts of the vitamin, the values ranging from 3.7 to 16.2 μg/day. Excretion of pantothenic acid for individual animals varied from week to week but showed no particular trends. Increasing the amount of ascorbic acid did not affect the concentration of pantothenic acid in the blood, liver or urine.

There was no correlation between blood pyruvic acid and blood pantothenic acid or intake of calcium pantothenate. Pyruvic acid values ranged from 1.86 to 4.54 mg/100 ml blood. At autopsy it was noted that the amount of visceral fat was related directly to intake of calcium pantothenate.

Although no attempt was made in the work presented here to duplicate the studies of Daft, the results reported do confirm the observations of Reid and Briggs (2); viz: that in the diet of the pantothenic acid-deficient guinea pig, large amounts of ascorbic acid appeared to be of no benefit. Reid and Briggs fed ascorbic acid at levels of 0 and 0.1 to 2.0% of the diet and reported that their animals were protected from scurvy even at the lowest level fed. Neither did any of the animals fed the lowest level of ascorbic acid (2 mg/day) in the present investigation display any symptoms of scurvy.

In diets containing suboptimal amounts of calcium pantothenate, Reid and Briggs reported that no benefit was derived from dietary ascorbic acid in excess of the amount needed to prevent scurvy. These workers considered calcium pantothenate fed at the level of 10 mg/kg of diet as suboptimal, 20 mg/kg as optimal and 40 mg/kg as an upper level, but reported no data on food consumption. If their animals consumed 9 to 15 g feed/day (based on consumption in this investigation), intake of calcium pantothenate was approximately 0.09 to 0.15 mg, 0.18 to 0.30 mg and 0.36 to 0.60 mg/day. These levels correspond with 0.06, 0.2 and 1 or 8 mg/day in the present study.

Whereas Reid and Briggs used a larger number of animals, and growth and survival as their principal criteria, the present investigators used a smaller number of animals but a larger number of criteria to arrive at essentially the same conclusions.

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Antimetabolites in the Nutrition of *Aedes aegypti* L. Larvae. Thiamine, Riboflavin, and Pantothenic Acid Antagonists

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ABSTRACT The effect of the antagonists of 3 B-vitamins on *Aedes aegypti* L. larvae, grown with an artificial diet, was studied. An inhibition index was computed for each of the antagonists. The ability of the vitamins to overcome the toxicity of the antagonists, and the growth-promoting effect of some vitamin analogues, which have vitamin-like activity in other organisms, were tested. Pyrithiamine was 40 times more toxic for mosquito larvae than oxythiamine. The toxicity of pyrithiamine was overcome by thiamine, although the inhibition index was lower with higher amounts of the vitamin in the diet. Galactoflavin was not toxic and had no riboflavin-sparing activity. Atabrine was toxic and its toxicity was independent of the amount of dietary riboflavin. Small amounts of atabrine slightly improved the rate of the larval development with diets containing suboptimal amounts of riboflavin. ω-Methylpantothenate was the only toxic pantothenic analogue. Its toxicity was not reversed by the vitamin. D-Pantothenyl alcohol and pantoyltaurine were not inhibitory. None of the pantothenic acid analogues had any vitamin-like activity. The effect of vitamin analogues in the nutrition of mosquito larvae is compared with that in other organisms.

Antimetabolites have proved to be a useful tool in insect nutrition and biochemistry. Antivitamins have been used for determining the vitamin requirements of insects when aseptic conditions or a chemically defined diet have not been available (1,2). Antimetabolites have also been used in the study of insect tissues in vitro (3), and of insect development (4). A new approach to insect control by the production of metabolic deficiencies has been suggested (5); antimetabolites, for example, have been used as control agents against insects feeding on textiles (6). The toxic effects obtained were attributed to vitamin deficiencies, although the antimetabolic action of the antagonists has not been proved. Detailed studies of the effect of antimetabolites on insects are needed to estimate their possible value in insect control. However, data on the quantitative vitamin-antivitamin relationship in insect nutrition are scarce. Our studies on the effect of antimetabolites in the nutrition of *Aedes aegypti* larvae, reared aseptically with an artificial diet, have shown that the mosquito larvae resemble higher animals in their response to choline (7), pyridoxine (8), and nicotinic acid antagonists (9). Vitamin analogues, such as 3-acetylpyridine and ω-methylpyridoxine, which have a vitamin-like activity for mammals, have shown a similar activity for mosquito larvae (8,9).

The present study is concerned with the effect of thiamine, riboflavin, and pantothenic acid analogues on *A. aegypti* larvae, grown with an artificial diet. We studied the relative toxicities of the better known antagonists and the ability of the vitamins to overcome their toxic effect. The vitamin-sparing activity of the riboflavin and pantothenic acid analogues, which have shown vitamin-like activity in other organisms, was also tested.

MATERIALS AND METHODS

Methods for rearing sterile *A. aegypti* larvae, for preparing the media and testing their sterility, and for evaluating the results have been reported previously (10). One larva was reared per tube, and its development recorded daily until it reached the adult stage or died. There were usually 10 replicates for each diet. The value of various diets was compared by using an index, which was obtained by dividing the

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sum of the percentage of larvae pupating plus the percentage of larvae reaching the adult stage by the median day of pupation. An index of about 25 was usually obtained with the basic diet (at 28°) and can be considered as optimal. Under these conditions up to 100% of the larvae reached the adult stage with a median larval period of 8 days. An index of zero was considered as complete inhibition, irrespective of the last instar reached by the larvae. The inhibition index was the ratio of moles of antimetabolite to moles of vitamin that results in 50% inhibition (11).

The standard diet (10) was based on solid vitamin-free casein (50 mg/tube) and 5 ml of liquid medium containing the following ingredients: (μg/ml) RNA,[1] 1000; inorganic salts,[2] 1450; cholesterol, 6; biotin, 0.1; folic acid, 0.6; thiamine·HCl, 2; riboflavin, 2; pyridoxine·HCl, 4; nicotinamide, 10; calcium pantothenate, 10; choline chloride, 100. In experiments with antagonists of thiamine, riboflavin, or calcium pantothenate the respective vitamin was omitted from the basic medium. The appropriate amount of thiamine, riboflavin, or calcium pantothenate, together with the antagonist tested, was added to the tube before the larva was introduced. All antimetabolites were obtained from commercial sources.

RESULTS

The first step was to determine the quantitative requirements of the larvae for

[1] Nucleic Acid Sodium Salt (Yeast), Mann Research Laboratories, Inc., New York 6.

[2] Inorganic salts contained the following: KH_2PO_4, 600; K_2HPO_4, 600; $FeSO_4 \cdot 7H_2O$, 12; $MnSO_4 \cdot 4H_2O$, 12; NaCl, 12; $CaCl_2$, 12; $MgSO_4 \cdot 7H_2O$, 200.

TABLE 1

Effects of thiamine·HCl, riboflavin, and calcium pantothenate on Aedes aegypti larvae

Vitamin[1]		No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index[2]
	μg/ml					
Thiamine·HCl	0	18	0			0
	0.02	22	0			0
	0.1	10	4	4	14	5.7
	0.15	13	11	11	9.5	17.8
	0.2	45	45	44	8	24.7
	2[3]	21	20	19	7.5	24.7
	20	22	22	21	7.5	26.1
	200	26	26	25	8	24.5
	1000	11	11	10[4]	7.5	25.4
	2000	32	31	24[5]	7.5	22.9
	4000	22	21	15	8.5	19.3
Riboflavin	0	32	0			0
	0.02	10	0			0
	0.1	20	4	3	42	0.8
	0.2	19	16	13	23	6.6
	0.6	9	9	9	11	18.2
	1.0	62	59	55	8.5	21.6
	2[3]	20	20	19	8	24.4
	10	22	22	22	8.5	23.5
	20	11	10	6	10	14.5
Ca pantothenate	0	36	0			0
	0.2	20	0			0
	1.0	22	9	6	20	3.4
	2	32	32	29	10	19.0
	4	53	52	47	8.5	22.0
	10[3]	25	25	25	8.5	23.5
	400	21	21	20	8.5	23.0
	2000	21	21	20	8.5	23.0
	4000	12	12	11	9	21.3

[1] Each vitamin tested was omitted from the standard basic diet and added in graded doses.

[2] Index = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.

[3] Amount of vitamin in the standard basic diet.

[4] One out of 10 adults incompletely emerged.

[5] Three out of 24 adults incompletely emerged.

TABLE 2

Effects of graded doses of antivitamins on Aedes aegypti larvae fed diets containing the minimal amount of the corresponding vitamin (thiamine·HCl, 0.2; riboflavin, 1; calcium pantothenate, 4 μg/ml)

Antivitamin added		No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index[1]
	μg/ml					
None		32	32	31	8.5	23.2
Pyrithiamine·HBr	10	19	18	16	9	19.9
	20	20	16	16	9.5	16.8
	100	23	9	5	15.5	3.9
	200	10	0			0
Oxythiamine chloride	10	11	11	10	9	21.2
	20	11	11	11	9.5	21.1
	100	12	12	10	10	18.3
	200	12	12	11	9.5	20.2
	1000	12	11	10	11.5	15.2
	2000	11	6	5	9	11.1
None		23	23	21	8	23.9
Galactoflavin	20	10	9	9	7.5	24.0
	50	10	10	10	7.5	26.7
	100	11	11	11	7.5	26.7
	200	17	13	12	7.5	19.6
Atabrine	20	10	10	10	9	22.2
	50	12	8	8	17	7.8
	100	11	5	5	28	3.2
	200	14	2	2	26	1.1
None		80	79	69	8.5	21.8
D-Pantoyl alcohol	200	7	7	7	8	25.0
	400	26	24	22	9	19.6
	1000	9	8	7	9.5	17.6
	2000	31	30	27	10	18.4
	4000	27	26	25	12	15.7
Pantoyltaurine	200	11	11	10	8.5	22.5
	400	18	18	17	9	21.6
	1000	17	16	16	8.5	22.1
	2000	17	16	16	9	20.9
	4000	12	12	12	9.5	21.1
ω-Methylpantothenate	200	17	17	16	8.5	22.8
	400	12	8	5	8.5	12.7
	1000	13	3	3	12	3.8
	2000	5	0			0

[1] Index = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.

thiamine·HCl, riboflavin, and calcium pantothenate. Each of the vitamins was omitted singly from the standard basic diet, and added in graded amounts (table 1). The minimal requirements for thiamine·HCl, riboflavin, and calcium pantothenate for normal development were 0.2, 1, and 4 μg/ml, respectively. These concentrations were used to test the effect of the antivitamins. According to Woolley (11) the antimetabolite-metabolite relationship is observed most clearly when enough metabolite is used to meet normal requirements.

The effect of excessive amounts of the vitamins was also tested, as large amounts of pantothenic acid are known to be highly toxic for house fly larvae (1). The highest amount of calcium pantothenate tested, which was 1000 times the minimum required, was not inhibitory for mosquito larvae. However, an excess of thiamine caused pupal mortality or incomplete emergence of some of the adults (see footnotes

4 and 5, table 1). Riboflavin was inhibitory when used in amounts 10 times that of the standard basic diet. The inhibition was mainly due to pupal mortality, but larval development was also retarded. The toxicity of riboflavin might be due to its low solubility; 20 μg/ml of riboflavin, which caused 40% inhibition, was the highest concentration of this vitamin that could be introduced into the diet in solution. Some of the riboflavin possibly precipitated on standing, and the larvae might have ingested much greater quantities of this vitamin than those in solution. Sodium salicylate could not be used to increase the solubility of riboflavin since it was toxic for the larvae (10). Thus the highest amounts of riboflavin and thiamine·HCl that could be used to overcome the toxicity of the antagonists, were 10 and 200 μg/ml, respectively.

The effect of graded doses of antivitamins, when added to diets containing the minimal amount of the corresponding vitamin, is shown in table 2. The relative toxicities of the 2 antithiamines differed widely. The inhibition index for pyrithiamine·HBr (mol wt 420) was approximately 250, and that for oxythiamine chloride (mol wt 340) was 10,000 (mol wt of thiamine·HCl, 337).

The toxicity of pyrithiamine for mosquito larvae could be overcome by increasing the amount of dietary thiamine (table 3). The antagonism, although reversible, was not competitive, since the inhibition index was lower in the presence of larger amounts of the vitamin (11). The inhibition index was 240 with diets containing minimal amounts of thiamine·HCl (0.2 μg/ml) and 120 with the standard basic diet (2 μg/ml thiamine·HCl). Lethal doses of pyrithiamine could be overcome by further increasing the amount of thiamine 10 or 100 times. With these diets less than one-half of the maximal inhibition occurred, and therefore the inhibition indexes could not be computed. Large amounts of pyrithiamine (250 and 500 μg/ml) impaired the emergence of adults; this was also observed in the presence of an excess of thiamine.

Galactoflavin and atabrine were tested with diets containing minimal riboflavin (table 2). The highest amount of galactoflavin that could be introduced into the diet in solution (200 μg/ml) caused only 20% inhibition, which was due to larval mortality. With more galactoflavin a precipitate was formed and all larvae died in the first instar. It is possible that with 200 μg/ml of galactoflavin some of it precipitated and was eaten by a larva. Thus the toxicity of galactoflavin could not be assessed quantitatively. Riboflavin itself was inhibitory at the limit of its solubility.

In the presence of 50 and 100 μg/ml of galactoflavin the index was slightly improved (table 2). Since small amounts of galactoflavin have a riboflavin-sparing activity for certain bacteria (12), we investigated whether galactoflavin spared riboflavin in the nutrition of mosquito larvae. No such effect was noted (table 4). Addition of 2 or 20 μg/ml of galactoflavin did not improve the riboflavin-deficient diets, whereas higher amounts of the analogue were inhibitory. The inhibition caused by 200 μg/ml of galactoflavin was greater than that expressed by our index, which does not take into account the viability of the adults, since some of the adults were unable to emerge normally (see footnotes 3, 4 and 5, table 4).

The antimalarial drug atabrine was very toxic for mosquito larvae. With diets containing minimal riboflavin (1 μg/ml) complete inhibition was obtained in the presence of 250 μg/ml of atabrine. The inhibition index, as calculated from table 2, was about 30 (mol wt atabrine, 509; mol wt riboflavin, 376). The toxic effect of atabrine for mosquito larvae could not be overcome by increasing the amount of dietary riboflavin 10 times. Atabrine, in concentrations ranging from 50 to 200 μg/ml was equally toxic with diets containing 1 or 10 μg/ml of riboflavin. The toxic effect of atabrine was most pronounced on the young larva. Many larvae died in the first or second instar, but those that survived reached the adult stage, although their development was very slow.

Low doses of atabrine improve the growth of young rats, receiving suboptimal amounts of riboflavin (13). The riboflavin-sparing activity of atabrine for mosquito larvae was therefore tested (table 5). A single non-toxic dose of atabrine (5 μg/ml) was added to diets deficient in

TABLE 3

Response of Aedes aegypti larvae to graded doses of pyrithiamine·HBr in the presence of different amounts of thiamine·HCl

Pyri-thiamine·HBr	Thiamine·HCl, μg/ml			
	0.2	2	20	200
μg/ml				
0	21.1[1](10)[2]	22.7 (10)	23.9 (11)	22.4 (10)
20	20.2 (11)	24.1 (14)	26.7 (14)	23.5 (9)
50	12.7 (7)	21.9 (12)	23.3 (12)	21.6 (11)
100	7.5 (12)	22.7 (10)	26.7 (11)	19.6 (12)
250	0 (8)	13.6 (11)	24.6[3](13)	21.4 (11)
500		1.8 (10)	18.2[4](11)	20.9[5](9)

[1] Values indicate index which = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.
[2] Figures in parentheses indicate number of larvae.
[3] Two out of 12 adults incompletely emerged.
[4] Six out of 7 adults incompletely emerged.
[5] Five out of 7 adults incompletely emerged.

TABLE 4

Effects of graded doses of galactoflavin on Aedes aegypti larvae fed riboflavin-free and low riboflavin diets

Riboflavin	Galactoflavin, μg/ml			
	0	2	20	200
μg/ml				
0	0[1] (6)[2]	0 (7)	0 (6)	0 (6)
0.1	0.9 (11)	0 (5)	0 (7)	0 (5)
0.2	6.1 (9)	7.6 (6)	3.7 (5)	0 (5)
0.4	12.0 (12)	15.2 (6)	10.0 (5)	9.8[3](6)
0.6	18.2 (9)	21.0 (5)	15.2 (5)	16.0[4](5)
0.8	22.2 (11)	25.0 (5)	22.2 (6)	20.0[5](5)
1.0	25.0 (9)	26.7 (8)	25.0 (6)	15.6 (5)

[1] Values indicate index which = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.
[2] Figures in parentheses indicate number of larvae.
[3] Four out of 5 adults incompletely emerged.
[4] Three out of 5 adults incompletely emerged.
[5] One out of 5 adults incompletely emerged.

TABLE 5

Effect of atabrine on Aedes aegypti larvae fed diets deficient in riboflavin

Riboflavin	Atabrine, 5 μg/ml	No. of larvae	No. of pupae	No. of adults	Median day of pupation	Index[1]
μg/ml						
0	—	16	0			0
0	+	15	0			0
0.25	—	30	21	19	24	5.6
0.25	+	31	25	23	19	8.1
0.5	—	31	28	27	15.5	11.5
0.5	+	32	29	26	11.5	14.9
1.0	—	20	18	18	9.5	19.0
1.0	+	10	9	9	8.5	21.2
2	—	10	10	9	8	23.7
2	+	11	11	10	8.5	22.5

[1] Index = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.

TABLE 6

Effects of pantothenic acid analogues on Aedes aegypti larvae fed diets deficient in calcium pantothenate

Ca pantothenate	Analogue added, 20 μg/ml			
	None	D-Pantothenyl alcohol	Pantoyl-taurine	ω-Methyl pantothenate
μg/ml				
0	0[1] (11)[2]	0 (10)	0 (13)	0 (10)
1	4.5 (10)	4.7 (11)	5.2 (10)	8.5 (11)
2	20.0 (11)	17.5 (12)	17.1 (10)	18.7 (9)

[1] Values indicate index which = $\frac{\%\text{ of pupae} + \%\text{ of adults}}{\text{median day of pupation}}$.

[2] Figures in parentheses indicate number of larvae.

riboflavin. With these diets larval development was slower than normal, but the percentages of pupae and of adults were not lower. Atabrine had no growth-promoting effect when added to the riboflavin-free diet. However, atabrine improved the rate of larval development when added to riboflavin-deficient diets. The mean day of pupation (the values in table 5 are those of the median day of pupation) with diets containing 0.25 μg/ml of riboflavin was 23.8 ± 0.68 (SE) without atabrine and 21.2 ± 0.86 when 5 μg/ml of atabrine were added. This improvement, although slight, was statistically significant ($P < 0.05$). The beneficial effect of atabrine was more obvious with diets containing 0.5 μg/ml of riboflavin. The mean day of pupation was lowered from 16.5 ± 0.76 (SE) to 12.4 ± 0.42 ($P < 0.01$). The addition of atabrine to low-riboflavin diets, however, did not make possible optimal development.

The effect of 3 pantothenic acid analogues on mosquito larvae, grown with diets containing a minimum of calcium pantothenate (4 μg/ml) was tested. Pantoyl taurine was not toxic for the larvae (table 2). D-Pantothenyl alcohol retarded larval development, but inhibition was less than 50%, as measured by the index, when the analogue was present in a concentration of 4 mg/ml. The only toxic pantothenic acid analogue was ω-methylpantothenate, which caused larval and pupal mortality, and slowed down development. The inhibition index was about 100.

The ability of calcium pantothenate to reverse the toxic effect of ω-methylpantothenate and the inhibition caused by D-pantothenyl alcohol was tested by increasing the amount of calcium pantothenate 500 times. Graded amounts of ω-methylpantothenate and D-pantothenyl alcohol, ranging from 200 to 4000 μg/ml, were added to diets containing 4 and 2000 μg/ml calcium pantothenate, respectively. Neither the toxic effect of ω-methylpantothenate nor the inhibition caused by pantothenol were reversed by the vitamin.

Finally, the ability of the pantothenic acid analogues to replace or spare the vitamin in mosquito larvae was investigated. D-Pantothenyl alcohol, pantoyl taurine and ω-methylpantothenate had no pantothenic acid sparing activity for mosquito larvae (table 6). The diets deficient in calcium pantothenate could not be improved by the addition of low, non-toxic doses of the 3 analogues.

DISCUSSION

Pyrithiamine, which is a competitive antagonist of thiamine in higher animals and in fungi and bacteria (11), requiring thiamine, had a similar effect in mosquito larvae and in house fly larvae (1). The relative toxicities of pyrithiamine and oxythiamine for insects are similar to those for higher animals and for yeasts and lactic acid bacteria. A single dose of pyrithiamine, but not of oxythiamine, is toxic when injected into silkmoth pupae (4). Pyrithiamine was 40 times more toxic for mosquito larvae than oxythiamine. The inhibition indexes in chickens, as computed from the growth response curves, are 4 for pyrithiamine and 200 for oxythiamine.[3] In *Kloeckera* and *Lactobacillus* the

[3] Naber, E. C., W. W. Cravens, C. A. Baumann and H. R. Bird 1954 Effect of thiamine analogs on embryonic development and growth of the chick. Federation Proc., 13: 469 (abstract).

inhibition indexes for pyrithiamine and oxythiamine were 4 to 40 and 1000 to 20,000, respectively (14). In mice both analogues inhibit growth but only pyrithiamine induces typical polyneuritis (11). Various explanations of the different toxicities of the 2 antithiamines have been offered (15).

No galactoflavin-riboflavin antagonism could be demonstrated in mosquito larvae, which were only slightly inhibited by a 200:1 galactoflavin:riboflavin ratio. Galactoflavin has not been tested in other insects. In chicks a galactoflavin:riboflavin ratio of 40:1 was also not inhibitory (12), but in rats galactoflavin caused a competitively inhibitory effect at a ratio of 50:1 (16). Small amounts of galactoflavin had some riboflavin-sparing activity for *Lactobacillus casei*, grown with a riboflavin-deficient medium, but no such effect is observed in chicks (12). Galactoflavin had no riboflavin-sparing activity for mosquito larvae.

The antimalarial drug, atabrine, has been found to be a competitive antagonist of riboflavin phosphate in enzymatic systems, and a growth inhibitor for *L. casei*. The latter effect could be overcome by increasing the riboflavin-content of the medium (17). Atabrine treatment in higher animals did not lead to a riboflavin-deficiency. On the other hand, low, non-toxic levels of atabrine improve the growth of young rats fed on a low riboflavin diet and increase urinary excretion of riboflavin in adult rats (13). Thus the same compound that competitively antagonizes riboflavin phosphate exerts a riboflavin-sparing effect. Atabrine shortened the larval period in mosquito larvae fed riboflavin-deficient diets. As in rats, atabrine exerted only a slight riboflavin-sparing action in mosquito larvae. The riboflavin-sparing action of atabrine in rats has been explained by the partial replacement of riboflavin by the structurally similar analogue in flavin-containing enzymes, or by the inhibition, by atabrine, of the metabolic destruction of the vitamin (13). Since a sparing effect was also observed in *A. aegypti* larvae, the same explanation may hold for that species.

According to Woolley (11), the action of an antagonist cannot always be overcome by the addition of the metabolite. When the harmful effect of an analogue of a vitamin is not reversed by the vitamin, it is difficult to decide whether the toxicity is due to the analogue's antimetabolic activity. In the case of galactoflavin and atabrine there was no evidence that these compounds act as riboflavin antagonists for mosquito larvae, and indeed they can hardly be considered as true riboflavin antagonists for these insects.

Since no specific signs of a pantothenic acid deficiency in mosquito larvae are known, the antimetabolic activity of ω-methylpantothenate could only be inferred by analogy. ω-Methylpantothenate is the only pantothenic acid analogue that causes a pantothenic acid deficiency in higher animals, including rats, mice, chicks (18), and humans (19). This analogue is the most toxic of all pantothenic acid analogues for several species of lactic acid bacteria (20).

Pantoyl taurine and pantothenol inhibit the growth of bacteria, but not that of higher animals (17). Pantothenol only slightly inhibited mosquito larvae, and pantoyl taurine had no inhibitory effect. Similarly, pantothenol and pantoyl taurine inhibited house fly larvae only when administered in high concentrations (1). Furthermore, pantoyl taurine is not toxic when injected into silkmoth pupae (4). Thus mosquito larvae, similarly to the other insects investigated, resemble higher animals, rather than bacteria, in their response to inhibitory analogues of pantothenic acid.

D-Pantothenyl alcohol, which is an effective substitute for pantothenic acid in warm blooded animals, but not in lactic acid bacteria (17), was ineffective in mosquito larvae.

The utilization of pantothenyl alcohol by animals depends upon its in vivo conversion to pantothenic acid. Mosquito larvae are apparently unable to perform this conversion.

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Dietary Protein Level and the Turnover Rate of Tissue Proteins in Rats

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ABSTRACT The effect of varying levels of dietary protein on the turnover rates of the combined proteins of several tissues in rats was studied. The rate of loss of isotope from the proteins of each tissue labeled following injection of DL-methionine-S^{35} was measured in young rats fed diets containing zero to 60% of casein for 3 weeks. The tissues examined were liver, kidney, plasma, spleen, intestinal mucosa, heart, muscle and testis. The apparent turnover rate of the proteins of each tissue was calculated from the slope of the response curve of the specific activity obtained plotting against the first, fourth and seventh day following injection of labeled methionine. Among these tissue proteins, the turnover rates of intestinal mucosa, plasma, kidney, spleen and liver proteins were affected by the protein level in the diet, and were increased with increments of the protein level. However, no further increase was observed by increasing the protein level above 25 or 40%. The response curves of the changes in turnover rates of these proteins plotted against the protein level were similar to that of body weight plotted in a similar way.

It is pertinent from the point of view of nutrition to determine the effect of dietary protein level on the turnover rate of tissue proteins. Solomon and Tarver (1) demonstrated that the rate of loss of methionine-S^{35} from the proteins of plasma, liver and kidney in rats was dependent on the protein content of the diet and that the rate of loss with a high protein diet was higher than that with a diet containing low protein. Steinbock and Tarver (2) measured the changes in half-life and replacement rate of the plasma protein in rats that occurred when the protein content of the diet changed. Jeffay and Winzler (3) have reported that the turnover rate of the S^{35}-labeled serum albumin in rats was dependent on the level of dietary protein, whereas the turnover rate of the S^{35}-labeled serum globulins was not. In contrast, recently Yuile et al. (4), using C^{14}-labeled plasma proteins in dogs, observed that the turnover rates of both plasma albumin and globulins were increased by increasing the level of dietary protein, but any further increase was not demonstrated with more than adequate protein level. Thus the results obtained by Yuile et al. differ somewhat from those reported by Steinbock and Tarver (2), and Jeffay and Winzler (3), in respect to the effect of increasing the level of dietary protein on the turnover rate of the total plasma protein, albumin and globulins. Furthermore, little information is available concerning the effect of dietary protein on the turnover rates of many other tissue proteins.

Muramatsu and Ashida (5, 6) have reported that several liver enzymes in rats responded to the varying levels of dietary protein differently, and that the response curves of some enzymes were correlated with those of the growth rates in animals. With respect to this problem, it appeared desirable to know whether any relationships may exist between the response curves of turnover rates of tissue proteins plotted against the level of dietary protein and that of the growth rates of animals plotted in a similar way.

The present study was, therefore, planned to investigate in detail the effect of varying levels of dietary protein on the rate of loss of isotope from the proteins of

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some tissues; liver, kidney, plasma, spleen, intestinal mucosa, heart, muscle and testis in rats injected intraperitoneally with S^{35}-labeled methionine. It was found that the rates of loss of radioactivity from the proteins of intestinal mucosa, plasma, kidney, spleen and liver increased with increasing levels of dietary protein, but did not further increase significantly with the protein levels above 25 or 40%, and that the response curves of their turnover rates plotted against the dietary protein level were similar to those of the growth rates in animals plotted in a similar way, as observed in the activities of some liver enzymes (5, 6).

EXPERIMENTAL

Animals and diets. Male weanling rats of the Wistar strain were fed a basal diet containing 25% of casein until they attained a body weight of 55 to 60 g. They were housed in individual cages in an air conditioned room maintained at approximately 25°C, and were fed the experimental diet and water ad libitum throughout the test periods. The experimental diets used, in which variations in the protein content were accomplished by substitution for α-starch, are shown in table 1. About 15 animals per group were used, except that 30 animals were used in a protein-free group.

Treatment of animals. After the rats had received the experimental diets for 21 days, all animals received intraperitoneally 2 μc of DL-methionine-S^{35} (3.0×10^6 count/min) per 100 g of body weight. They were then placed in metabolism cages, and killed on the twenty-second, twenty-fifth and twenty-eighth day of the experiment, i.e., on the first, fourth and seventh day after injecting methionine-S^{35}. All rats were anesthetized with ether and killed by withdrawing blood from the heart.

Preparation of samples. Blood was collected in a centrifuge tube containing 0.2 ml of 1% sodium heparinate solution, and the plasma was separated by centrifugation. Liver, kidney, spleen, intestinal mucosa, heart, muscle and testis were rapidly removed. They were frozen at − 20°C in a deep freezer, and aliquots were homogenized in a glass homogenizer with a Teflon pestle using cold 10% TCA. In animals fed a protein-free diet, tissues of each of the 2 rats were pooled and homogenized. Heart and muscle were dissolved in 0.4 N NaOH prior to above treatment (7).

The labeled proteins of various tissues were prepared by the usual method (8), then digested with perchloric-nitric acid (Piries' reagent). Sulfur was precipitated as benzidine sulfate, filtered and dried; then its radioactivity was measured on filter paper with a windowless gas-flow Geiger counter. Corrections were made for background, self-absorption and radioactive decay. The benzidine sulfate was then titrated with 0.01 N NaOH in a boiling solution, using phenol red indicator. The results were expressed as specific activity (counts per minute per milligram of sulfur).

Correction of specific activity. In the growing animals the observed decrease in the specific activity with time following injection of labeled methionine is due not only to the turnover of the protein, but also to the dilution effect from an increase in the total protein as the animal increases in body size. Jeffay (9) has shown that in the determination of serum proteins in growing rats the actual change in pool size must be considered. Therefore, to obtain the correct turnover rate of proteins of each tissue, the dilution effect due to the increase of protein content must be eliminated. An estimation of the corrected specific activity was made according to the following equation.

$$\text{Corrected s.a.}_t = \frac{\text{observed s.a. at time}_t \times \text{total protein content at time}_t}{\text{total protein content on the first day following injection of labeled methionine}}$$

where t = time of day following injection of methionine-S^{35}.

Corrected s.a._t = expected specific activity corrected for increase of protein content at time_t.

TABLE 1
Composition of experimental diets

	%	%	%	%	%
Casein	0	10	25	40	60
Sesame oil	5	5	5	5	5
Salts mixture[1]	5	5	5	5	5
Vitamin mixture[1,2]	0.25	0.25	0.25	0.40	0.60
α-Starch	89.6	79.6	64.6	49.4	29.2

[1] Harper, A. E., J. Nutrition, 68: 405, 1959.
[2] The diet also contained per 100 g: 0.15 g of choline chloride (1 ml in 50% ethanol) and 0.07 ml of a mixture containing vitamin A, 30,000 IU and vitamin D_2, 3,000 IU/ml (Chokora A, Eisai Company, Ltd., Tokyo).

The correction should be made essentially for zero time, but for convenience, was made for the first day following injection of labeled methionine. No correction was made for the muscle proteins. Protein was determined by the method of Lowry et al. (10).

RESULTS

The rates of decrease of the specific activity in proteins of each tissue in this study are shown in figure 1. The curves in this figure were drawn by plotting the average data for the specific activity of proteins of each tissue against the time following injection of DL-methionine-S^{35}.

In the preliminary experiments, we observed that the specific activities of proteins of each tissue following the intraperitoneal administration of labeled methionine reached a maximum at about one to 8 hours, after which it showed a gradual decline. However, no significant changes in muscle were observed throughout 7 days following injection of the isotope. These results were not significantly different from those of Tarver et al. (11, 12), who observed the same reactions by administering methionine-S^{35}, orally (11) or intravenously (12). In addition, it was found that the time required for reaching the maximal radioactivity in liver proteins was not markedly altered in each group fed diets containing varying levels of protein. From these preliminary experiments, it was assumed that on the first day the level of the S^{35} labeling in several tissues would reach the maximal value. Therefore, the radioactivity in tissue proteins was measured on the first, fourth and seventh day following injection of methionine-S^{35} in order to determine the rate of loss of the labeling in tissue proteins, i.e., the turnover rate.

Figure 1a indicates that the level of the labeling of liver proteins was highest in rats receiving a protein-free diet, and lower as the protein level of the diet was increased. Nearly the same value was observed for the 40 and the 60% casein group. Similar results were observed in kidney and intestinal mucosa. Figure 1a also shows that the rates of decrease of the specific activity of the liver proteins in rats of all groups were nearly linear throughout the duration of the experiment, and the rate of decrease was slowest with a protein-free diet and became greater as the level of the dietary protein increased from zero to 25%, but did not greatly change with the protein levels above 25%. Changes in the specific activity of the methionine sulfur in the liver proteins of rats fed diets containing different dietary protein levels using the procedure in which the cystine sulfur was removed as the cuprous mercaptide (13), were also determined. No significant differences were observed between these results and those in figure 1a.

The rate of loss of radioactivity of the kidney proteins in rats receiving diets containing varying levels of casein was similar to that of the liver proteins (fig. 1b). However, each initial level of the specific activity on the first day was slightly higher than that observed in the liver proteins.

The rate of decrease of the specific activity of the intestinal mucosa proteins in each group was higher than that of the liver and kidney proteins in each corresponding group (fig. 1c). In this tissue, the slope of the decline was not linear over the entire period, showing that there are at least 2 groups of proteins having different turnover rates in this experimental condition. The rate of loss of specific activity in these tissue proteins with a pro-

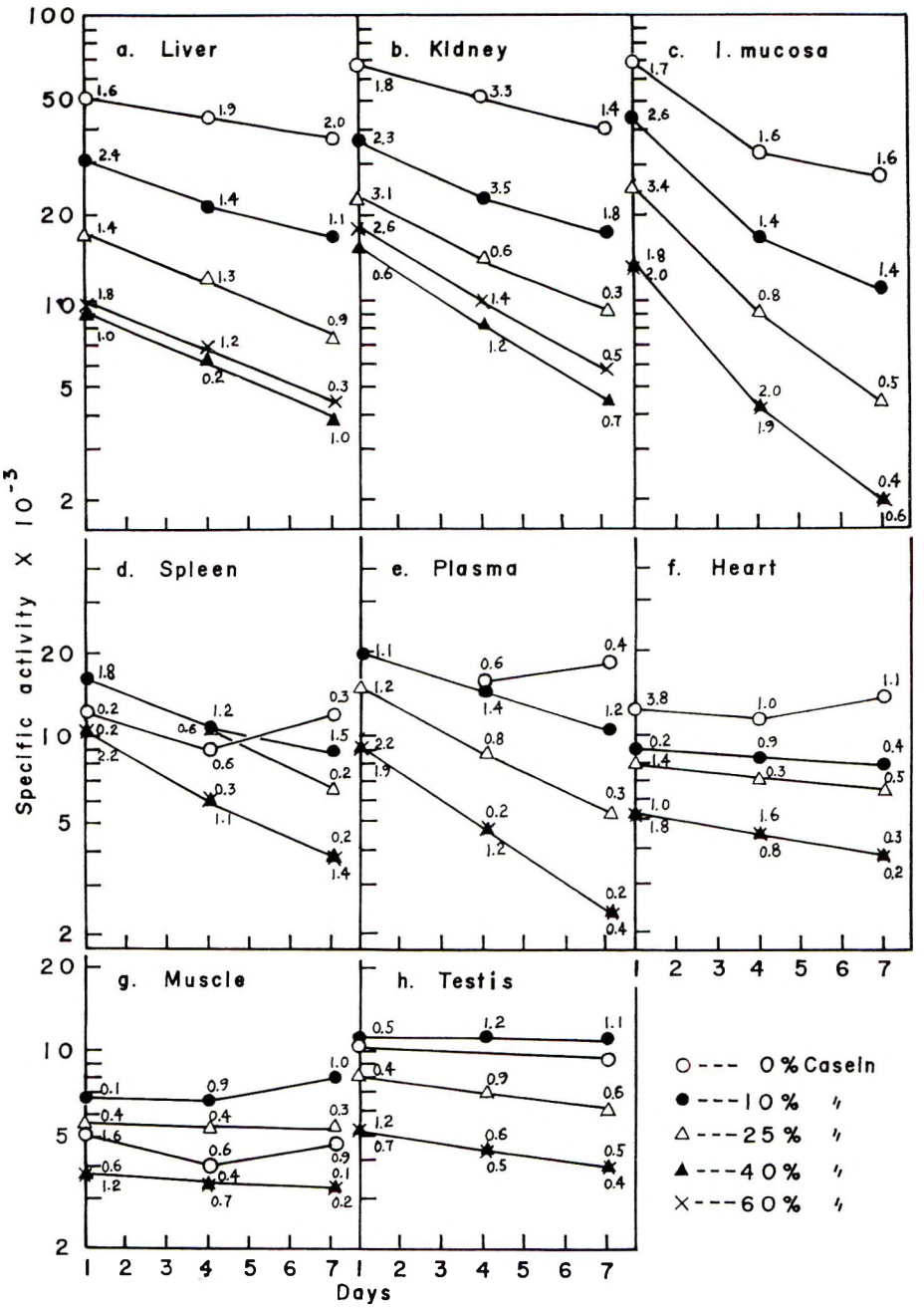

Fig. 1 Specific activities of the proteins of liver, kidney, intestinal mucosa, spleen, plasma, heart, muscle and testis in rats fed diets containing varying levels of casein. The curves are drawn by plotting the mean of the specific activity on the semilog section paper against the time following injection of DL-methionine-S^{35}. Vertical lines represent specific activity (count/min/mg of sulfur of protein). Horizontal lines represent days after injection of the radioisotope. Values of each point represent standard error of the mean ($\times 10^{-3}$). Four to 5 rats were used for each point.

tein-free diet and the 10% casein diet was less than that with the 25% or more casein diets, as observed in the liver and kidney proteins.

The specific activities of the spleen proteins in rats fed a protein-free diet were decreased from the first to the fourth day, followed by a slight increase from the fourth to the seventh day (fig. 1d). The initial levels of the specific activity of the spleen proteins on the first day following injection of the radioisotope in each group did not differ to the same extent as those of the intestinal mucosa, kidney and liver proteins, and the specific activity levels of zero and 10% casein groups were lower than those of the intestinal mucosa and kidney proteins in each corresponding group. Similar observations were also noted in the other tissues such as plasma, heart, muscle and testis (fig. 1e–1h). In these tissues, furthermore, the slope of the specific activity with a protein-free diet tended to remain relatively constant during the entire period under consideration, or to increase slightly from the fourth to seventh day of the experiments. In heart and testis tissues, the rate of loss of specific activities failed to show large changes among all groups fed diets containing varying levels of casein. Therefore, the effect of dietary protein level on the rate of loss of radioactivity in heart and testis proteins was less clear. Muscle tissue proteins appear to show the least change in the rate of loss of the specific activity.

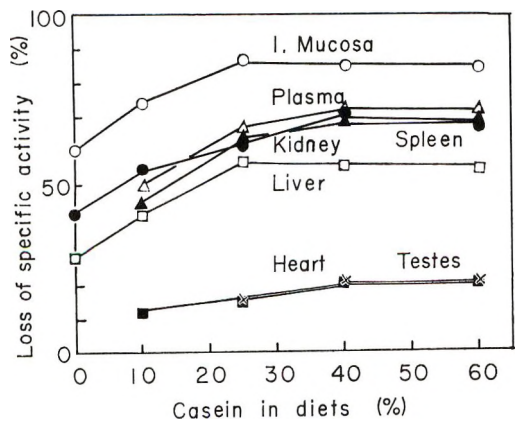

Fig. 2 Changes in the percentage decrease of specific activities of some tissue proteins of rats fed diets containing varying levels of casein.

Figure 2 was obtained by plotting the percentage decrease of the specific activity in proteins of each tissue during the period from the first to seventh day against the dietary level of protein. This figure indicates that the rate of loss of the label from the proteins in several tissues such as intestinal mucosa, plasma, kidney, spleen and liver, increased with increasing levels of casein in this diet, but no further increase was observed with the protein levels above 25 or 40%, and that the pattern of these changes resembled those of the body weight as shown in figure 3.

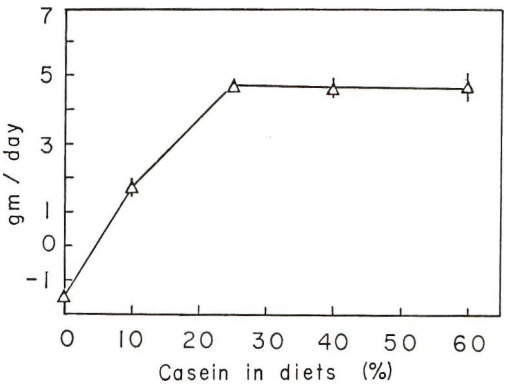

Fig. 3 Changes in body weight of rats fed diets containing varying levels of casein. Vertical lines represent standard error of the mean.

DISCUSSION

The radioactivity levels of the proteins in intestinal mucosa, kidney and liver on the first day following injection of methionine-S^{35} in each group were higher than, or the same as, those of the other tissues examined in each corresponding group. In all of the above mentioned tissues the radioactivities in the proteins were higher in rats that received lower protein diets than in those fed protein-rich diets. Similar observations have been made by several workers, in rat liver (1), in rat plasma (14), in rat serum and liver (15), in rat visceral organs including liver (16), in dog serum (17). Sidransky and Farber (18), and Nimni and Bavetta (19), feeding rats a threonine- or tryptophan-deficient diet, respectively, also reported that the specific activity of liver and serum proteins for the deficient group was higher than that for the supplemented group. The lower spe-

cific activities, on the first day, of intestinal mucosa, kidney and liver proteins in rats that received the high protein diets are considered to be due to the greater dilution of the label with methionine in dietary protein, as suggested by Solomon and Tarver (1). In spleen, plasma, heart, testis and muscle, on the other hand, the specific activities on the first day did not differ with varying dietary protein levels as much as those in intestinal mucosa, kidney and liver, even though these tended to be lower as the protein level in the diet increased. Accordingly, it is considered that the labeling in these tissues was not affected to any extent by variations in the methionine of dietary origin.

The slope of the response curve of the specific activity obtained by plotting against the first, fourth and seventh day following injection of labeled methionine will reflect the apparent turnover rate of the proteins of each tissue. The average apparent half-life of proteins of some tistues in rats fed the 25% casein diet, which were estimated from the slope of the response curve of the specific activity, were as follows: intestinal mucosa, about 2.0 to 2.9 days; plasma, 3.3 to 4.4 days; spleen, 4.1 days; kidney, 4.1 to 4.9 days; liver, 5.0 days. The half-life of heart, muscle and testis proteins could not be calculated, since the changes in the specific activities of these tissue proteins during 7 days were not significant. These values do not necessarily indicate the true turnover rates, because the re-utilization of the label would occur. It may be considered, however, that the apparent turnover rates of some tissue proteins calculated above would be close to the true value, since it has been shown by Goldworthy and Volwiler (20) that re-utilization of the isotope does not become significant until about 4 weeks after intravenous injection of labeled plasma protein into dogs fed an adequate diet. The half-life values of liver and plasma protein have been estimated by many workers, showing 2.8 to 7 days for the former protein (11, 12, 21, 22) and 3 to 5 days for the latter one (2, 4, 23, 25). Thompson and Ballou (22) demonstrated the existence of labile and also inert components in nearly all tissues. The value for the turnover rate of proteins of each tissue in this study would belong to that of the dynamic component in tissue.

The results shown in figure 2 indicate that the apparent turnover rates of some tissue proteins increased with increasing the protein level in the diet, and those of intestinal mucosa and liver proteins reached a maximum with about 25% casein level, and those of kidney, plasma and spleen proteins reached a maximum with about a 40% casein level, but no further increase occurred with the protein levels above 25 or 40%. These results are in agreement with those of Yuile et al. (4), who showed that the rate of plasma protein turnover was more rapid in dogs receiving adequate dietary protein than in those receiving a deficient protein diet; and that when increments were made to this adequate dietary protein level, no further increase in turnover occurred. However, present data differed somewhat from those reported by Solomon and Tarver (1), Steinbock and Tarver (2) and Jeffay and Winzler (3), who observed that the turnover rate of liver, total plasma and serum albumin protein in rats increased with increasing the levels of dietary protein. To confirm our results, we made a re-investigation on the turnover rates of the liver proteins in rats receiving the 25% or more protein diets (26). The results showed that the turnover rate of liver proteins was slightly increased as the casein level in diets was increased from 25 to 60%. However, the increases were much less than those in the turnover rate of liver proteins with increasing the casein level from zero to 25%.

Previous reports indicated that the feeding of the lower protein diets that did not produce maximal growth resulted in the decrease of liver nitrogen and of some liver enzyme activities such as xanthine oxidase and succinic dehydrogenase in rats (5, 6). Comparison of the present results with the previous results leads one to consider that there may be a correlation between the pattern of the growth response and the response curves of the turnover rate of some tissue proteins or those of liver xanthine oxidase because the maximal growth was obtained in rats receiving about 25% casein diet, at which level the turnover rate of some tissue proteins or

the activity of liver xanthine oxidase reached each maximal value.

The decreased turnover rate of tissue protein might be considered as an occurrence accompanied by the decreased decomposition of nucleic acid. In that case, this may be a reason that there is a correlation between the response curve of liver xanthine oxidase which participates in the catabolism of the purine bases of nucleic acid and that of the turnover rate of tissue protein.

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